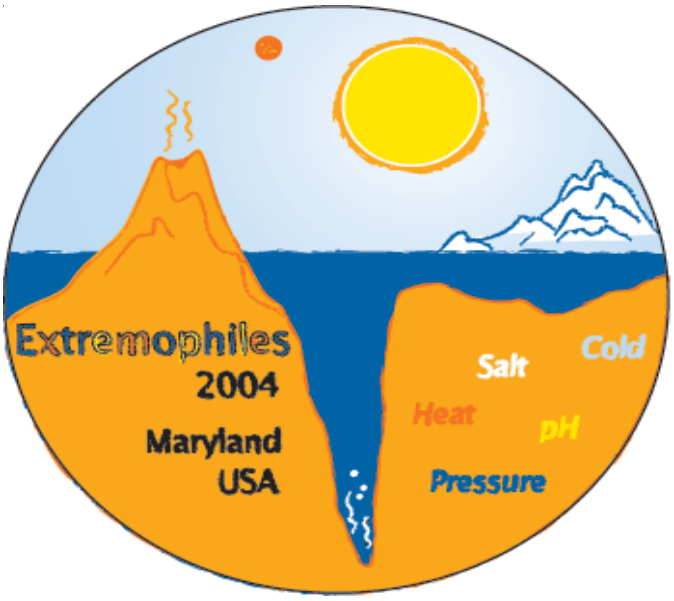

Extremophiles 2004

5th International Conference on Extremophiles

SEPTEMBER 19 -23, 2004

CAMBRIDGE, MARYLAND

AMERICAN
SOCIETY FOR
MICROBIOLOGY

Extremophiles 2004

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To identify topics of scientific significance which encourage interactive exchange in meetings of 100 to 700 people.

To encourage student and postdoctoral participation in these focused areas.

To recruit individuals in disciplines not already involved in ASM to ASM membership.

To budget this program to break even with an emphasis on meeting ASM member needs.

REGISTRATION

During the conference, the registration desk will be located in the foyer of the Chesapeake Ballroom. ASM staff will be available to assist you during session hours.

ORAL SESSIONS

All oral sessions will be held in the Chesapeake Ballroom. Concurrent sessions are in divided sections of the Chesapeake Ballroom, as noted on the program schedule. A name badge is required for entry into all sessions. In consideration of other participants, no children are permitted in the sessions.

POSTER SESSIONS

Poster boards are located in the 2nd level conference rooms: Clipper, Galleon, Cutter and Schooner. Presenters should mount posters on Sunday evening and should leave posters up for the entire conference. Posters should be removed after the afternoon session on Wednesday, no later than 5 pm. Each poster is allotted half of a board space. Please check your assigned number in the abstract index and mount your poster on the board space bearing that number.

Official poster sessions will be held on Monday, Tuesday and Wednesday as noted within the program. Please present your poster during the appropriate poster sessions (Odd posters present during odd numbered poster sessions; even posters present during even numbered poster sessons).

Posters are grouped by topic area as follows:

DNA Replication	Posters 1 - 11
Gene Expression	Posters 12-33
Genetics and Genomics	Posters 34-55
Industrial/Biotechnology	Posters 56-93
Origins/Exobiology/Ecology	Posters 94-147
Physiology and Metabolism	Posters 148-191
Structural Biology/Enzymology	Posters 192-230
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MEALS

The Welcome Reception will be held on Sunday, September 19, in the Regatta Pavilion (outdoors). Breakfast and lunch will be provided in the Choptank Ballroom on Monday, Tuesday and Wednesday. Breakfast will be provided Thursday morning. The Conference Dinner will be held at the River's Edge (outdoors) on Wednesday, September 22, and is included in the registration fee. Tickets for guests for the conference banquet may be purchased at the registration desk for US$50.

STUDENT TRAVEL GRANTS

ASM encourages the participation of graduate students and new postdocs at ASM Conferences. To support the cost of attending the conference, ASM has awarded travel grants of $500 to each of the following individuals:

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Elif Yavuz

SUNDAY 19 SEPTEMBER

19:30 -19:40
Chesapeake Ballroom

Welcome and Introduction
Frank Robb

19:40-20:30
Chesapeake Ballroom

Opening Lecture: Chasing Vibrios: From the Deep Sea to the Bay of Bengal
Rita Colwell

20:30-22:00
Regatta Pavilion (outside)

Welcome Reception

MONDAY 20 SEPTEMBER

7:00-8:30
Choptank Ballroom

Breakfast

8:30-9:45
Chesapeake Ballroom

Plenary Address: NEPTUNE: A Next-Generation, Interactive Earth-Ocean Research Platform for Microbial Ecology
John Delaney, *Introduction by Anna Louise Reysenbach*

9:45-10:00

Break

10:00-12:00

Concurrent Sessions

Chesapeake A/B/C/D

Session 1A: Origins/Evolution/Diversity I
Chair: Anna Louise Reysenbach

New Thermophilic Prokaryotes With Different Types of Anaerobic Respiration
Liza Bonch-Osmolovskaya

Novel Microorganisms from the Microbial Observatory Uzon Caldera in Kamchatka, Russia
Juergen Wiegel

10:00-10:30

Break

Microbiology of Subglacial Environments
Brian Lanoil

Living Microbes on Silt Grains in Greenland Basal Ice at 3053 m Depth
H. Camas Tung

Chesapeake E/F/G

Session 1B: Gene Expression
Chair: Michael Thomm

Purification, Characterization and Crystallization of RNA Polymerase from Hyperthermophlie Archaeon *Pyrococcus furiosus*
Katsuhiko Murakami

The Trehalose/Maltose System of *Thermococcus litoralis*; Transport Enzymes and Regulation
Winfried Boos

10:00-10:30 Break

Pyrrolysine Activation for Protein Synthesis
Dieter Söll

Identification of Binding Sites for the Transcriptional Regulator Ss-LrpB in the Genome of *Sulfolobus solfataricus* P2
Daniel Charlier

12:00-14:00
Choptank Ballroom

New Frontiers and Technologies Luncheon Colloquium
sponsored by Takara Bio, Japan
Cold Shock High Expression Vectors and Single Protein Production in Living Cells
Masayori Inouye

14:00-17:00 Concurrent Sessions

Chesapeake A/B/C/D

Session 2A: Industrial/Biotech I
Chair: Francine Perler

New Nucleic Acid Processing Enzymes from Thermophilic Bacterial and Viral Genomes
Jakob Kristjansson

Extremophilic Archaea and Bacteria as Source of Hydrolases of Industrial Interest
Garo Antranikian

Applied Biotechnology in the Oil Industry
Hans Christian Kotlar

15:30-16:00 Break

Complete Genome Analysis and Application of the Hyperthermophilic Archaeon, *Thermococcus kodakaraensis* KOD1
Tadayuki Imanaka

Thermostable Enzymes from a Randomized Hybrid Library
Mike Finney

Chesapeake E/F/G

Session 2B: Structure Function Relationships I
Chair: Rudolf Ladenstein

What Molecular Simulations Can Tell Us About Protein Stability in Extreme Conditions
Adrian Elcock

Structural Adaptations of Psychrophilic Enzymes
Charles Gerday

A Proteomic Investigation of *Methanosarcina acetivorans* Cultured with Acetate and Methanol
James Ferry

15:30-16:00 Break

The Crystal Structure of an Extraordinary, Open Pore Ferritin from the Hyperthermophilic Archaeon *Archaeoglobus fulgidus*
Imke Schröeder

Catalysis with a Pinch of Salt
David Rice

17:00-18:00 Poster Session 1

18:00-20:00 Dinner Break (on your own)

20:00-22:00 Poster Session 2

TUESDAY 21 SEPTEMBER

7:00-8:30 Breakfast
Choptank Ballroom

8:30-12:00 Concurrent Sessions

Chesapeake A/B/C/D **Session 3A: Extremophile Metabolic Enzymology I**
Chair: Douglas Clark

Multienzyme Complexes In The Extreme: The 2-Oxoacid Dehydrogenase Complexes Of The Archaea
Mike Danson

Specialized Roles of the Two Pathways for the Synthesis Of Mannosylglycerate in Osmoadaptation and Thermoadaptation of *Rhodothermus marinus*
Nuno Borges

Genomics-based Functional Analysis of the Central Carbohydrate Metabolism of *Thermoproteus tenax*
Bettina Siebers

10:00-10:30 Break

Stabilization by Cyclization - RNA and Protein Engineering Exercises
John Van der Oost

Biogenesis of Sugar Binding Proteins in *Sulfolobus solfataricus*
Arnold Driessen

Special Features of the ATP Synthase are Required for Oxidative Phosphorylation by Alkaliphilic Bacillus at High pH
Terry Krulwich

Chesapeake E/F/G

Session 3B: Origins/Evolution/Diversity II
Chair: Daniel Prieur

Metabolic Diversity of Halophilic Microorganisms: Bioenergetic Constraints Affecting the Functioning of Hypersaline Ecosystems
Aharon Oren

Novel Families of Archaeal Viruses from Hot Acidic Springs in Pozzuoli, Italy
David Prangishvili

Phage Communities in Hot Springs and Other Extreme Environments
Forest Rohwer

10:00-10:30 Break

Deep-sea Piezophiles, Their Ecology, Genomes, and Physiology
Chiaki Kato

Microbial Diversity in a Solar Saltern
Carol Litchfield

Solar System for Exobiology: Instrumentation for Detection of Evidence of Extraterrestrial Life
Edward Sittler Jr.

12:00-14:00
Choptank Ballroom

Future Directions In Extremophile Research Luncheon Panel Discussion
R. Kelly (Chair) M. Moracci, M. Noordewier, G. Antranikian, H. Morgan, P. Dennis, K. Horikoshi, D. Clark

14:00-15:00
Chesapeake Ballroom

Plenary Address: From Giants to Dwarfs: Perspectives on the Discovery of Hyperthermophiles
Karl Stetter, *Introduction by John Reeve*

15:00-15:30
Chesapeake Ballroom

International Society for Extremophiles General Membership Meeting
Chair: Koki Horikoshi

15:30-20:00 Free Time

20:00-22:00 Poster Session 3

WEDNESDAY 22 SEPTEMBER

7:00-8:30
Choptank Ballroom
Breakfast

8:30-12:00
Concurrent Sessions

Chesapeake A/B/C/D

Session 4A: DNA Replication
Chair: Simonetta Bartolucci

Envisioning Dynamic Molecular Machines Acting in Maintenance of the Genome
John Tainer

Function, Structure and Evolution of the Replication Protein from the Archaeal Plasmid pRN1
Georg Lipps

Molecular Mechanisms of Progression and Repair of the Replication Fork in Archaea
Yoshizumi Ishino

10:00-10:30
Break

The *Methanothermobacter thermautotrophicus* MCM Helicase
Zvi Kelman

The DNA Replication Machinery of the Hyperthermophilic Archaeon *Sulfolobus solfataricus*
Steve Bell

Genetic Identification of Archaeal DNA Replication Origins from *Haloferax volcanii*
Thorsten Allers

Chesapeake E/F/G

Session 4B: Structure Function Relationships II
Chair: Jennifer Littlechild

The Expanding Universe of Small Non Coding RNAs in the Hyperthermophilic Archaeon, *Sulfolobus solfataricus*
Patrick Dennis

Oxidation of Inorganic Sulfur Compounds in *Acidianus ambivalens*
Arnulf Kletzin

Structure Anaylsis of the Heat Shock Regulator from *Pyrococcus furiosus*
Rudolf Ladenstein

10:00-10:30
Break

Archaeal Protein Translocation Systems
Mechtchild Pohlschroeder

Cryo-Electron-Tomography of *Thermotoga maritima*: Chemoreceptor Cluster and the Flagellar Motor
Ariane Briegel

Molecules and Structures Involved in the Cell-Cell Interaction Between *Ignicoccus* and *Nanoarchaeum*
Reinhard Rachel

12:00-14:00
Choptank/Poster Area

Lunch and Poster Session 4

14:00-17:00

Concurrent Sessions

Chesapeake A/B/C/D

Session 5A: Functional Genomics
Chair: Marco Moracci

The Genome Sequence of the Thermoacidophilic Archaeon *Picrophilus torridus* and its Implications for Metabolic and Molecular Adaptation to Life Around pH 0
Wolfgang Liebl

A Systems Approach to Elucidate DNA Repair Mechanisms and Regulation in the Archaea
Jocelyne DiRuggiero

Insight into Cold Adaptation in Archaea from Genomic and Proteomic Studies
Rick Cavicchioli

15:30-16:00

Break

Comparative Analysis of *Thermotoga* species
Karen Nelson

Stress Responses of *Pyrococcus furiosus*
Mike Adams

Functional Genomics of the Model Halophile *Halobacterium* sp. NRC-1
Jochen Mueller

Chesapeake E/F/G

Session 5B: Extremophile Metabolic Enzymology II
Chair: Jenny Blamey

Physiological and Ecological Significance of Glycoside Metabolism in Hyperthermophilic Heterotrophs
Robert Kelly

Evidence that a Phosphomannomutase from *Sulfolobus solfataricus* is Regulated by Phosphorylation
Peter Kennelly

New Extreme Thermophiles from High Temperature Compost
Tairo Oshima

15:30-16:00 Break

The Role of Coenzyme A disulfide Reductase and Small Intracellular Thiols in the Metabolism of *Pyrococcus*
Edward Crane

Activity-Stability Relationships in Extremophilic Enzymes
Salvino D'Amico

The First Crystal Structure of Hyperthermostable NAD-dependent Glutamate Dehydrogenase from *Pyrobaculum islandicum*
Toshihisa Ohshima

18:00-22:00
The River's Edge (outdoors)
Conference Dinner, Announcement of Extremophiles 2006 and International Society for Extremophiles Awards Presentation

THURSDAY 23 SEPTEMBER

7:00-8:30
Choptank Ballroom
Breakfast

8:30-10:30 Concurrent Sessions

Chesapeake A/B/C/D
Session 6A: Industrial/Biotech II
Chair: Tadayuki Imanaka

Enhanced Expression of a Thermophilic Bacterial Xylanase (XynB) in *Trichoderma reesei*
Peter Bergquist

Re-designing Life *In-Silico*
Donald Ward

Characterization of Thermophilic Microbial Consortia Associated with Oil
Krista Kaster

Biological Neutralization of Highly Alkaline Textile Waste Water Using an Extremophile, *Exiguobacterium sp.* (DSM ID 03-501)
Anil Kumar

Pressure Perturbation Calorimetric Studies of Bipolar Tetraether Liposomes Derived from the Thermoacidophilic Archaeon *Sulfolobus acidocaldarius*
Parkson Chong

Denaturation of an Extremely Stable Tetrameric Hyperthermophilic Protein
Sara Lawrence

Chesapeake E/F/G

Session 6B: Genetics
Chair: Kevin Sowers

Extreme Stability of a Sulfolobus Genome: Experimental Measurement and Mechanistic Implications
Dennis Grogan

Global Regulation by Hydrogen Limitation in *Methanococcus maripaludis*
John Leigh

Molecular Analysis of a *Haloferax volcanii* HMG CoA Reductase Mutant Strain
Kelly Bidle

Genome of the Halophilic Bacterium *Salinibacter ruber*
Emmanuel Mongodin

Functional Genomics of Low Temperature/High Pressure Adaptation
Doug Bartlett

Exploring the *Sulfolobus solfataricus* Proteome by Two Dimensional Electrophoresis and LC-LC-MS-MS
Ambrosius Snijders

10:30-10:45 Break

10:45-11:30 Poster Talks: Award Winners

1130-11:45 Closing Remarks

11:45 Conference Concludes

PA:1

NEPTUNE: A NEXT-GENERATION, INTERACTIVE EARTH-OCEAN RESEARCH PLATFORM FOR MICROBIAL ECOLOGY

***J. R. Delaney**; University of Washington, Seattle, WA.*

The earth, ocean, planetary, and biological sciences are in a transformational period triggered by the confluence of technological advances in sensor technologies, robotic systems, high-speed communication, nanotechnology, and dramatically escalating capabilities to computationally simulate reality. The NEPTUNE regional cabled ocean observatory capitalizes on these advances and is part of a worldwide effort to develop coastal, regional, and global ocean observatories. NEPTUNE's 3200-km network of fiber-optic/power cables will encircle and cross the Juan de Fuca tectonic plate in the northeast Pacific Ocean, an area roughly 500 km by 1,000 km in size. Approximately 25 experimental sites will be established at laboratory nodes distributed across the submarine network. This novel facility will deliver high-bandwidth telecommunication capabilities and considerable electrical power to thousands of sensor systems and many autonomous vehicles distributed over thousands of square kilometers of seafloor and extending throughout the full ocean depths. The ultimate vision of NEPTUNE is to enable routine, real-time interaction between an extensive community of land-based users and sets of diverse *in situ* instrumental sensor arrays. These arrays will be comprised of remotely operated, user-generated experiments that will detect and quantify variability over a wide range of spatial and temporal scales for a broad range of ocean and earth processes. Sensor networks, interactive experiments, and data archives will be accessible to researchers, educators, students, policy makers, and the public around the globe via Internet This next-generation system will function as a prototype for new types of remote natural laboratories to enable a spectrum of microbial and ecological studies throughout the ocean basin.

S1A:1

NEW THERMOPHILIC PROKARYOTES WITH DIFFERENT TYPES OF ANAEROBIC RESPIRATION

***E. A. Bonch-Osmolovskaya**; Institute of Microbiology, Russian Academy of Sciences, Moscow, RUSSIAN FEDERATION.*

Oxidation of non-fermentable substrates in the course of anaerobic respiration constitutes an important step in the biogeochemical cycling of elements in hydrothermal systems. With molecular hydrogen as a substrate, such respiration could support the growth of lithoatotrophs responsible for primary production of organic matter in anoxic thermal ecosystems. Anaerobic oxidation of acetate, one of the major products of anaerobic organic matter destruction, closes the anaerobic carbon cycle in thermal environments. By isolating sulfur-, nitrate- and iron-reducing thermophilic prokaryotes on both these substrates, a wide diversity of these organisms was shown. The new sulfur reducers inhabiting deep-sea hot vents in the Pacific and Atlantic Oceans represent the novel order *Nautiliales*. They are obligate hydrogen-oxidizing chemolithoautotrophs associated with indigenous deep-sea polychaeta populations. The genus *Nautilia* comprises obligate sulfur-reducers, while the members of the genus *Caminibacter* can use sulfur or nitrate as electron acceptors, or grow microaerobically. The ability to grow via nitrate reduction was also found in diverse new thermophilic bacteria isolated from deep-sea thermal habitats. Two new microaerophilic members of the *Thermaceae* family representing the novel genera *Oceanithermus* and *Vulcanithermus* were found to use hydrogen, acetate, and many other non-fermentable substrates in the course of nitrate reduction to nitrite. *Caldithrix abyssi* representing a novel deep phylum in Bacteria Domain was shown to be capable of both fermentation of organic substrates and anaerobic respiration by oxidizing hydrogen or acetate and reducing nitrate to ammonium. A novel thermophilic bacterium *Thermoincola ferrireducens* isolated from hot springs of the Kunashir Island (Kurils) was able to oxidize hydrogen, acetate, and peptides in the course of Fe(III) reduction. Radioisotopic experiments with ^{14}C-labeled acetate and HCO_3^- and different electron acceptors confirmed the results of the cultivation approach, but also revealed the existence of microorganisms not obtained so far in laboratory cultures.

S1A:2

NOVEL MICROORGANISMS FROM THE MICROBIAL OBSERVATORY UZON CALDERA IN KAMCHATKA, RUSSIA

***J. Wiegel**; Univ. of Georgia, Athens, GA.*

A unique interdisciplinary, international microbial observatory to explore the exciting microbial world in remote volcanic and geothermal areas of the Kamchatka Peninsula, Siberia (Russia) has been established in 2003. The geological situation of Kamchatka is unique, due to the unusual concentration of active volcanoes and geothermal areas at a low altitude leading to higher boiling temperatures in the springs compared to the US Yellowstone Natl. Park. Especially the features of Uzon Caldera and Geyser Valley are different from the geothermal features at Yellowstone National Park. Unusual biogeochemical and mineralogical processes have been described in the Uzon Caldera, and the microbial communities is should be different from other geothermal areas with different environmental conditions. The presence of many novel microorganisms is indicated by preliminary results, i.e., by already described and by now newly isolated microorganisms. We will present a short overview on the diversity of microorganisms already isolated and present then some environmental 16S rRNA sequence analysis of highly arsenic and antimony, mercury and sulfur containing pools (e.g., including from the Arkashin Shurf, a small bright yellow-orange pool) and compare these to the heterotrophic and autotrophic isolates we have obtained so far. Furthermore we will present our attempts to correlate the isolates to the wide range of geological and detailed geochemical data obtained during last year from these pools to obtain a new

biogeochemical concept how the microbial community depends on / is influenced by the geochemistry of the pools but more importantly also to elucidate whether and how the microorganisms influence the geochemistry of the individual pools.

S1A:3

MICROBIOLOGY OF SUBGLACIAL ENVIRONMENTS

***B. Lanoil**; University of California, Riverside, CA.*

Until recently, subglacial environments were thought to be abiotic. However, a growing body of literature indicates the presence of abundant and active subglacial microbial communities that significantly influence solute composition and flux from alpine and Arctic glaciated basins and continental ice sheets. We compared chemical and microbiological parameters for two glaciers—a temperate valley glacier (Bench Glacier, Alaska: BG) and a polythermal Arctic glacier (John Evans Glacier, Ellesmere Island, Nunavut, Canada: JEG). Despite similar solute composition, the geochemistry of the two systems is quite different. The primary weathering reactions at BG are sulfide oxidation coupled to carbonate dissolution with contributions by carbonate hydrolysis; at JEG they are gypsum dissolution and carbonate hydrolysis. Based on 16S rRNA gene similarity to nearest neighbors in public databases, the predicted physiologies of dominant microbial community members appears to correlate with the dominant chemical weathering processes in these two systems: BG has abundant clones related to iron or sulfide oxidizers, while at JEG they are rare. Based on quantitative dot blot hybridization to 16S rRNA gene PCR products, these patterns are stable over time, and the subglacial microbial community at both sites is distinct from supraglacial or ice marginal communities. Intriguingly, it appears that the dominant phylogenetic groups present in these systems are also found in sediment samples from beneath Ice Stream Kamb, West Antarctic Ice Sheet, perhaps indicating a selection for these organisms by the cold, dark and isolated conditions found in all subglacial environments. This is the first non-culture based study of the ice/water/sediment interface of subglacial environments. These data provide further support for the concept that microbes are significant players in geochemical cycling in subglacial environments.

S1A:4

LIVING MICROBES ON SILT GRAINS IN GREENLAND BASAL ICE AT 3053 M DEPTH

***H. C. Tung**, N. Bramall, P. B. Price; University of California, Berkeley, Berkeley, CA.*

Two ice cores 30 km apart extended ~3050 m down to bedrock at Summit, Greenland. Most attention has been focused on their high-resolution climate record covering the last ~110,000 years. In the bottom few meters, which contained up to 0.65 wt % of silt (mean diameter 3 μm), R. Souchez *et al.* found a factor of 500 excess of CO_2 and a factor of 10^4 excess of CH_4 in the GRIP basal ice, and T. Sowers measured a factor 2 x 10^4 excess of CH_4 at 3043 m in the nearby GISP2 basal ice. V. Miteva *et al.* discovered ~7 x 10^7 microbial cells/ml in Sowers' sample. Souchez *et al.* inferred from the huge excesses that the ice formed by mixing with a peat deposit. It is now clear from our work and the observations of Miteva *et al.* that the excesses were the direct products of *in-situ* microbial metabolism. The basal ice, with its silt and microbial population, likely formed before the ice sheet developed. Using DAPI stain and epifluorescence, we analyzed the relationships of microbes to silt grains at six depths from 3044 to 3053 m in GISP2 ice. Microbial concentrations ranged from ~10^7 to >10^9/cm^3 of ice and correlated with weight fraction of silt rather than with height. Between 92% and 99% of the microbes were attached to silt particles at a typical concentration of ~10^7/cm^2 of grain surface. This correlation extended down to the smallest silt grains: typically, up to 10 cells were attached to a 2 μm grain. With an SEM we found that some cells were dividing, and we were able to culture microbes in low-nutrient liquid media. These two results demonstrated their viability after 10^5 yr at -9°C.

S1B:1

PURIFICATION, CHRACTERIZATION AND CRYSTALLIZATION OF RNA POLYMERASE FROM HYPERTHORMOPHILE ARCHAEON PYROCOCCUS FURIOSUS

*A. Hirata, **K. S. Murakami**; The Pennsylvania State University, University Park, PA.*

Recent structural results have provided unprecedented insights into the structure and function of RNA polymerases (RNAPs) from virus, bacteriophage, bacteria and eukaryote, but not from archaebacteria. However, the archaeal RNAP could be the best target for high resolution X-ray crystal structure determination in multi-subunit RNAP family (bacteria, eukaryote and archaea), because; 1) the archaeal, but not the bacterial, basal transcription machinery resembles the eukaryotic RNAP II (Pol II) apparatus, 2) only the structure of the archaeal preinitiation complex (PIC) is seems to be able to be solved by the X-ray crystallographic study because of the complexity (more than 20 polypeptides) and the size (over 2 MDa) of eukaryotic PIC, and 3) only modest resolutions of the eukaryotic transcription elongation complexes (TECs) have been reported. We have established purification protocol of transcriptionally active *Pyrococcus furiosus* (*Pfu*) RNAP from *Pfu* cells. The *Pfu* RNAP is able to form stable transcription elongation complex (TEC) with DNA/RNA scaffold and the TEC is capable of extending the RNA in sequence specific manner. The crystallization screenings of the *Pfu* RNAP and the TEC are in progress.

S1B:2

THE TREHALOSE/MALTOSE SYSTEM OF THERMOCOCCUS LITORALIS; TRANSPORT, BIOCHEMISTRY, STRUCTURE AND REGULATION

***W. Boos**; University of Konstanz, GERMANY.*

Thermococcus litoralis contains a gene cluster of about 16 kb that appears in nearly identical sequence in Pyrococcus furiosus. It contains the genes for a binding protein-dependent ABC transporter specific for trehalose and maltose. TrmB, contained within the same cluster encodes the maltose/trehalose-specific gene regulator for the operon acting as repressor. The operon contains in addition treT encoding trehalose synthase a novel enzyme that catalyzes the reversible transfer of glucose from ADP-glucose to glucose to form trehalose. Most likely, the enzyme is not involved in the synthesis but the degradation of trehalose after its accumulation from the medium. TrmB, the specific repressor of the system is acting as global regulator serving several operons depending on the sugar that is bound to it. This is particular evident for the control of the maltodextrin transport operon that is specifically repressed by TrmB but its repression is only relieved by maltotriose but not by maltose or trehalose. In contrast, the maltose/trehalose transport genes that are specifically repressed by TrmB are relieved from repression by maltose or trehalose but not maltotriose. TrmB also binds sucrose with high affinity (in competition with trehalose and maltose), but binding of sucrose does not result in the induction of either operon. The structure of MalK, the ATP-hydrolyzing subunit of the maltose/trehalose transporter will be discussed in its potential role of gene regulation.

S1B:3

PYRROLYSINE ACTIVATION FOR PROTEIN SYNTHESIS

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Ribosomal protein synthesis requires twenty sets of aminoacyl-tRNAs, one for each canonical amino acid. It was commonly accepted that all organisms possess twenty aminoacyl-tRNA synthetases, each enzyme specific for attaching one amino acid to tRNA. However, biochemical and functional genomic studies in bacteria and archaea have overturned this concept in the past few years. It is now clear that the biosynthetic routes to asparaginyl-, glutaminyl-, lysyl- and cysteinyl-tRNA vary greatly in many organisms. Instead of direct aminoacylation that takes place in eukaryotes, amide aminoacyl-tRNA (Asn-tRNA and Gln-tRNA) formation in most prokaryotes is catalyzed by transamidation of Asp-tRNA^{Asn} and Glu-tRNA^{Gln}. Even more unexpectedly, the synthetic mechanism of Gln-tRNA synthesis is not conserved in evolution, but differs in the three domains of life. A further complexity is seen in Asn-tRNA and Gln-tRNA synthesis in bacteria, where at least four different scenarios are in use. The heteromeric transamidation enzymes that catalyze Asn-tRNA or Gln-tRNA formation in bacteria and archaea evolved by recruitment of amino acid metabolizing enzymes. This possible evolutionary link between protein synthesis and amino acid biosynthesis is further highlighted by the discovery that tRNA-dependent amidation of aspartate appears to be the sole route to asparagine synthesis in many bacteria and archaea. While cysteinyl-tRNA formation in a number or methanogenic archaea is still unknown, the presence of two classes of lysyl-tRNA synthetases in *Methanosarcineae* may be related to pyrrolysine incorporation.

Ibba, M. and Söll, D. (2004) Aminoacyl-tRNAs: setting the limits of the genetic code. *Genes Dev.* **18**, 731-738.

S1B:4

IDENTIFICATION OF BINDING SITES FOR THE TRANSCRIPTIONAL REGULATOR SS-LRPB IN THE GENOME OF SULFOLOBUS SOLFATARICUS P2

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Most of the best studied archaeal transcriptional regulators belong to the Lrp family (Leucine responsive regulatory protein), yet little is known about their physiological role and significance (1). Recently we have identified Ss-LrpB (Sso2131) from S. solfataricus as a new member of this family of bacterial/archaeal regulators (4). Ss-LrpB is most similar to Ptr2 from Methanococcus jannaschii (33% aa sequence identity), a transcriptional activator of ferredoxin and rubredoxin genes (3), and to LrpA from Pyrococcus furiosus (32% identity) of which the 3D crystal structure has been solved but its function is still unknown (2). Ss-LrpB binds cooperatively to three similar and regularly spaced targets in its own control region with as consensus the 15 bp palindrome -5'- TTGYAWWWWWTRCAA - 3'. In our search for the role of Ss-LrpB we have screened in silico the genome sequence of S. solfataricus P2 for the presence of similar Ss-LrpB box sequences occuring in intercistronic regions. Different control regions appear to show different numbers of binding sites and various organizations, also with respect to the promoter elements. Some of the most promising potential binding sites, present in front of the porDAB cluster (pyruvate ferredoxine oxidoreductase subunits, Sso2128-2130), and of the mtaP (5'-methylthioadenosine phosphorylase, Sso2343) and Sso0049 (conserved hypothetical protein) genes have been amplified and cloned. In vitro binding of purified recombinant Ss-LrpB was demonstrated by electrophoretic mobility-shift assay (EMSA). The detailed characterization of these promoter/operator regions and their interaction with Ss-LrpB is in progress. We will report on the characterization of the various Ss-LrpB-DNA complexes by DNase I and 1,10-phenanthroline-copper ion in gel footprinting and on the determination of transcription start points and transcript levels.

(1) Brinkman, A.., Ettema, T., de Vos, W. & van der Oost, J. (2003). Mol. Microbiol. 48, 287-294.
(2) Leonard, P., Smiths, S.., Sedelnikova, S., Brinkman, A., de Vos, W., van der Oost, J. & et al. (2001). EMBO J. 20, 990-997.
(3) Ouhammouch, M., Dewhurst, R., Hausner, W., Thomm, M. & Geiduschek, E. (2003). Proc. Natl. Acad. Sci. 100, 5097-5102.
(4) Peeters, E., Maes, D. & Charlier, D. This Book of Abstracts.

S2A:1

NEW NUCLEIC ACID PROCESSING ENZYMES FROM THERMOPHILIC BACTERIAL AND VIRAL GENOMES

J. K. Kristjansson[1], *S. H. Hjorleifsdottir*[1], *T. Blondal*[2], *A. Aevarsson*[1], *O. H. Fridjonsson*[1], *S. Skirnisdottir*[1], *S. Ernstsson*[1], *G. O. Hreggvidsson*[2]; [1]*Prokaria Ltd, Reykjavik, ICELAND,* [2]*University of Iceland and Prokaria Ltd., Reykjavik, ICELAND.*

In order screen for and identify novel nucleic acid modifing enzymes we have used two major approches. One is the well established whole genome sequencing of thermophiles of both bacterial and viral origins by using standard shotgun DNA sequencing. From our viral genome sequencing project, we have identified several thermostable counterparts of nucleic acid modifing enzymes including a RNA ligase from a *Thermus scotoductus* bacteriophage TS2126 and a polynucleotide kinase from *Rhodothermus marinus* phage RM378. The RNA ligase shows high ligation activity at 50-75°C, including high ssDNA ligation properties which is a very interesting property for use in molecular biology. The polynucleotide kinase shows also high 5'-kinase activity at 50-80°C on both RNA and DNA and presents new phosphohydrolase domain unknown in polynucleotide kinases known today. In our DNA polymerase discovery project we use gene retrieval by GENEMINING™ methodology from unidentified biomass as a high throughput discovery platform. We have used GENEMINING™ methodology to clone, express and screen for thermostable DNA polymerases with new, interesting properties. Data from discovery and characterisation of novel DNA polymerases displaying interesting new properties which will also be presented.

S2A:2

EXTREMOPHILIC ARCHAEA AND BACTERIA AS SOURCE OF HYDROLASES OF INDUSTRIAL INTEREST

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Although numerous reactions have been performed using enzymes from mesophiles, there is still a challenge to identify and exploit novel biocatalysts from extremophiles. A variety of extremophiles including thermo-, psychro-, acido- and alkaliphiles were investigated for their ability to metabolize fats, oils, carbohydrates, proteins and amides. A highly stereoselective amidase, which was able to hydrolyze a broad range of aliphatic, aromatic, heterocyclic and amino acid amides was purified and characterized. A number of lipases, which were identified from strict anaerobic microorganisms, were successfully expressed in mesophilic hosts such as E. coli and yeast. Due to their unique regio- and enantioselectivity such enzymes are of value for numerous applications. Furthermore, data will be presented on the properties of a recombinant glucoamylase from Picrophilus torridus and the protease from Fervidobacterium islandicum.

S2A:3

APPLIED BIOTECHNOLOGY IN THE OIL INDUSTRY: INTRODUCING BIOTECHNOLOGY ACROSS THE VALUE CHAIN IN AN OIL COMPANY WITH SPECIAL EMPHASIZE ON THE USE OF EXTREMOPHILES

H. K. Kotlar*, O. G. Brakstad, S. Markussen, A. Winnberg; Statoil.*

Although microbial enhanced oil recovery (MEOR) is a relatively familiar method – biotechnology has generally been underexploited by the oil industry. However, this situation is rapidly changing. Experts are investigating the use of bacteria as cost-effective, self-generating, biochemical factories whose environment-friendly products can substitute traditional chemicals and catalysts and create spin-off business opportunities. Potential use of extremophile organisms is of great interest. Be aware, though, that this is an immature area with more questions than answers. Biodiversity and biological activities in oil reservoirs are still poorly understood. There are fundamental issues regarding energy pathways and reaction rates. Nobody knows just how far organisms or their genetic information can be used for these purposes. Nevertheless, Statoil is going ahead with "applied biotechnology" as part of its New Trends and Opportunities R&D program.
This is an immensely wide area, so we have chosen to focus on three main areas:

- ***Preventive medication***: using these methods to create environment-friendly biochemicals for preventing formation damage, sand production and the precipitation of multiphase flow-blocking phenomena
- ***Bioreactors:*** developing enzyme or bacterial systems to replace traditional catalysts used to upgrade oil quality and/or generate clean bio-energy.
- ***Extremophiles:*** examining the potential of bacteria living under extreme conditions (of temperature, pressure etc.) for such applications and new product development.

Could specifically designed bacteria be used as mini-refineries, or introduced during transport and storage to remove crude oil penalty products, such as S, V, Ni and N-compounds? The field is wide open.

S2A:4

COMPLETE GENOME ANALYSIS AND APPLICATION OF THE HYPERTHERMOPHILIC ARCHAEON: THERMOCOCCUS KODAKARAENSIS KOD1

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Thermococcus kodakaraensis KOD1 is a hyperthermophilic archaeon isolated from a solfatara on Kodakara Island, Kagoshima, Japan. The strain grows optimally at 85°C and pH6.5, and can utilize a variety of organic compounds as carbon source such as starch, amino acids and pyruvate. We have recently determined the

2,088,737 bp complete genome sequence of strain KOD1, and have also developed a gene disruption system in order to elucidate the *in vivo* functions of individual genes. Further, the expression levels of all genes are being examined under various conditions utilizing a DNA chip of *T. kodakaraensis*. In this paper, I will introduce the features of various genes and proteins that are of particular interest in terms of application. DNA polymerase from strain KOD1 exhibits rapid elongation, high fidelity and high processivity, and has been commercially available as a high performance PCR enzyme. DNA ligase is not only a thermostable enzyme, but displays unique cofactor dependency at elevated temperatures. We have also examined a ribulose 1,5-bisphosphate carboxylase/oxygenase (*Tk*-Rubisco) in this strain. *Tk*-Rubisco displayed higher activity and higher carboxylase specificity than previously reported Rubiscos. The enzyme also displayed a unique $(L_2)_5$ decameric pentagonal structure, which was found to be necessary for the high thermostability of the enzyme. Another interesting aspect of *T. kodakaraensis* is the presence of a vast array of polysaccharide degrading enzymes. Among the starch-degrading enzymes, thermostable á-amylase, 4-á-glucanotransferase and cyclodextrin glucanotransferase have been characterized. The strain also harbors a novel, chitin-degradation pathway composed of chitinase, exo-â-D-glucosaminidase and diacetylchitobiose deacetylase. The chitinase possessed endo- and exo-type catalytic domains together with three chitin-binding domains on a single polypeptide, while the structures of the latter two enzymes were novel. We have also purified and characterized a Ni-Fe hydrogenase from *T. kodakaraensis*. Growth of the strain on pyruvate or starch in the absence of elemental sulfur led to high production rates of molecular hydrogen in the gas phase. Conditions of continuous cultures are being optimized with the strain, and in addition, attempts to improve the production rates via a metabolic engineering approach are being initiated.

S2A:5

THERMOSTABLE ENZYMES FROM A RANDOMIZED HYBRID LIBRARY

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To create thermostable DNA polymerases with new properties, we took the approach of generating a randomized hybrid-protein library. We started with the sequences of two family B thermostable DNA polymerases, which we call the "parental sequences". The parental sequences were aligned, showing 115 differences out of 775 amino acids, or 85% identity. For each of the non-identical positions, a degenerate DNA sequence was designed, wherever possible, so that both of the parental amino acids would be encoded. Degenerate oligonucleotides were synthesized and used to construct a library where each member of the library encodes the parental amino acid at each of the positions where the parental sequences are identical, while encoding one or the other of the parental amino acids, randomly, at nearly all of the positions where the parental sequences differ. Fifty-four random clones expressing full-length protein were selected. Of these, forty-eight (89%) had detectable thermostable polymerase activity. Of the six non-functional clones, the two with the highest expression levels have been sequenced, and both contain unintended mutations expected to impair polymerase activity. We have not yet observed any clones with the intended hybrid sequences that do not result in thermostable polymerase activity. As expected, we have obtained polymerases with variable properties, including exonuclease-to-polymerase activity ratios and salt preferences. A hybrid polymerase with low exonuclease-to-polymerase activity ratio has been fused with the thermostable DNA-binding domain Sso7d from Sulfolobus solfataricus to enhance polymerase processivity (Wang et al., NAR 32, 1197-1207, 2004). The resulting polymerase is capable of PCR-amplifying long sequences (e.g. 40 kb) with high efficiency and high fidelity.

S2B:1

WHAT MOLECULAR SIMULATIONS CAN TELL US ABOUT PROTEIN STABILITY IN EXTREME CONDITIONS

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Molecular dynamics (MD) simulations provide an atomic-level view of the structure and dynamics of molecular interactions in aqueous solution. In this talk, the basis of the simulation method will be briefly summarized before I describe the results of work aimed at understanding the effects of extreme conditions on molecular associations. Recently published studies of the effects of high temperatures on the thermodynamics of amino acid interactions will be outlined first, followed by a presentation of unpublished work examining the effects of very high NaCl concentrations (up to 3M) on electrostatic and hydrophobic associations.

S2B:2

STRUCTURAL ADAPTATIONS OF PSYCHROPHILIC ENZYMES

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Psychrophilic enzymes display two main properties :a high specific activity at low temperature and a high thermosensitivity. Moreover ,their specific activity is in general lower than that of their mesophilic counterparts at the respective environmental temperatures meaning that the adaptation could not be complete. These enzymes face in fact two challenges that of the temperature and also of the high viscosity of aqueous environments at low temperature both tending to drastically reduce the rate of biochemical reactions. To understand the adaptation to cold the structural properties of thirteen enzymes families were investigated in order to try to define some general rules regarding cold adaptation at the molecular level. Another aim of the study was to try to rationally transform a cold adapted enzyme into a highly thermostable one. For this purpose a cold adapted alpha –amylase was selected; the enzyme displays as usual a high specific activity at low temperature and a high thermosensitivity as demonstrated by fluorescence spectroscopy and differential scanning calorimetry. Its thermal unfolding is reversible and occurs through a single transition enabling the determination of

the stability curve showing that the maximal stability is reached at 17°C. Calculation of the enthalpic and entropic contributions shows that at low temperature the enzyme is stabilized thanks to a favourable entropy term while the enthalpy term is unfavourable. Thermal inactivation occurs well below the melting temperature illustrating the lability of the active site or of the enzyme substrate complex. Numerous mutants were produced by introducing amino acids found in more stable alpha –amylases. The data show that the adaptation to cold is due to an increase of the overall plasticity of the molecular edifice as demonstrated by fluorescence quenching using acrylamide. The increase in flexibility is generated through a weakening of the intramolecular interactions leading to a thermal instability which approaches its limits .Selected mutations give rise to an enzyme displaying properties not far away from that of the mesophilic counterpart using only five mutations. A second strategy was discovered in the case of enzymes such as chitobiase and phosphoglycerate kinase interacting with small size substrates. In these cases only one domain shows a low stability influencing the flexibility of the active site whereas other domains show on the contrary an increase stability when compared to the mesophilic homologue presumably in order to keep a good affinity of theses enzymes for the substrates .

S2B:4

THE CRYSTAL STRUCTURE OF AN EXTRAORDINARY, "OPEN PORE" FERRITIN FROM THE HYPERTHERMOPHILIC ARCHAEON *ARCHAEOGLOBUS FULGIDUS*

***I. Schroeder**, E. Johnson, D. Cascio, M. Sawaya; UCLA, Los Angeles, CA.*

Ferritins are important iron storage/detoxification proteins found ubiquitously in living organisms. Despite low sequence homology between species, the 3-dimentional structure of known ferritins is highly conserved. All ferritins previously characterized are tetracosameric (comprised of 24 subunits) and assemble to form a hollow, largely closed spherical shell with 4-3-2 symmetry. This report details the 2.1 Å resolution structure of the ferritin from the hyperthermophilic Archaeon *Archaeoglobus fulgidus*. The *A. fulgidus* ferritin (AfFtn) monomer has a high degree of structural homology with known prokaryotic and eukaryotic ferritins (r.m.s. deviation of ~ 0.9 Å), but the 24 subunit biological assembly represents the only known ferritin which packs to form a shell with tetrahedral (3-2) rather than octahedral (4-3-2) symmetry. The result of this unique packing configuration is a shell containing 4 large (~ 45 Å) pores with threefold symmetry that arrange in a tetrahedral configuration. . Here we present details of the AfFTNs subunit fold, metal binding center, quaternary structure and compare its unique tetracosameric architecture with that of archetypal ferritins. Finally we offer a hypothesis explaining why the AfFTNs unique structure is appropriate for it to function properly in the physiological context of an anaerobic and hyperthermophile.

S2B:5

CATALYSIS WITH A PINCH OF SALT

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The structure of glucose dehydrogenase from the extreme halophile *Haloferax mediterranei* has been solved at 1.6Å resolution under crystallisation conditions which closely mimic the "*in vivo*" intracellular environment to reveal details of the protein/solvent interactions which contribute to salt tolerance. The structure shows that the protein is surrounded by a multi-layered hydration shell which is more extensive and highly ordered than that observed for almost any other protein structure solved to date. Molecular features responsible for this include the decoration of the enzyme's surface with acidic residues, which are only partially neutralised by five bound potassium counter-ions, and a significant reduction in exposed hydrophobic surface, arising from a decrease in the proportion of lysines in the amino acid sequence of the enzyme. The latter is consistent with the global reduction of this residue type in the genomes of halophiles. Surprisingly, despite the preponderance of negative charges on the surface of the protein, the majority of ligands to the bound potassium ions are carbonyl oxygens of the protein main chain. The recognition of the 2' phosphate of the adenine ribose and the pyrophosphate moiety of the NADP involves a cation cluster, formed by two potassium ions which are stabilised by water-mediated interactions with an acidic C-terminal tail of the protein. The utilisation of such a counter-ion cluster may well represent a novel adaptation to high salt that allows a reduction in the proportion of exposed lysine residues.

S3A:1

MULTIENZYME COMPLEXES IN THE EXTREME: THE 2-OXOACID DEHYDROGENASE COMPLEXES OF THE ARCHAEA

***M.J. Danson,** H.C. Aase, C. Heath, D. Al Mailam, A.C. Jeffries, D.W. Hough; Centre for Extremophile Research, University of Bath, Bath, UNITED KINGDOM.*

Aerobic Bacteria and Eukarya possess a family of 2-oxoacid dehydrogenase multienzyme complexes that catalyse the general reaction:

2-Oxoacid + CoASH + NAD^+ Acyl-SCoA + CO_2 + NADH + H^+

Members of this family include the pyruvate dehydrogenase complex (catalyses the conversion of pyruvate to acetyl-SCoA), the 2-oxoglutarate dehydrogenase complex (2-oxoglutatarate to succinyl-SCoA) and the branched chain 2-oxoacid dehydrogenase complexes (oxidatively decarboxylate the 2-oxoacids produced by the transamination of amino acids valine, leucine and isoleucine). Thus, these enzymes serve catabolic roles in the pathways of central metabolism (Perham, 1991). The complexes are all three component systems consisting of multiple copies of enzymes E1 (2-oxoacid decarboxylase), E2 (dihydrolipoyl acyl-transferase) and E3 (dihydrolipoamide dehydrogenase). E2 forms the structural core of the complex, to

which copies of E1 and E3 are non-covalently bound. The number of copies of each component can vary between the different complexes, although the E2 core comprises either 24 or 60 polypeptide chains. E2 also forms the catalytic core of these multienzyme complexes, with each E2 polypeptide chain having a covalently-bound acyl-carrying cofactor, lipoic acid, which serves to connect the three active sites and to channel substrate through the enzyme complex. In contrast to this situation, no 2-oxoacid dehydrogenase complex activity has ever been found in the Archaea, the equivalent reactions being catalysed by much simpler ferredoxin oxidoreductase enzymes. However, in this presentation we will provide evidence that the aerobic Archaea do indeed contain a 2-oxoacid dehydrogenase complex:

- E3 enzymic activity has been found and characterised in *Haloferax volcanii, Halobacterium halobium, Thermoplasma acidophilum* and *Aeropyrum pernix*.
- Lipoic acid has been detected in *Hbt. halobium* by GC-MS.
- An operon, comprising 4 genes that show high sequence identities with those encoding bacterial E1a, E1b, E2 and E3 components of 2-oxoacid dehydrogenase complexes, has been discovered in *Hfx. volcanii*, *Hb. NRC1*, *T. acidophilum*, *Sulfolobus solfataricus* and *A. pernix*.
- Structural predictions on the protein sequences, and conservation of catalytic residues, suggest that the proteins are functional components of a 2-oxoacid dehydrogenase complex.
- The 4 genes of the operon from *T. acidophilum* have been cloned into an expression vector and the proteins expressed in soluble form. The E2 is not lipoylated by the *E. coli* host, but characterisation of the E1 component shows it to catalyse the oxidative decarboxylation of branched chain 2-oxoacids.
- *In vivo* transcription of the complete operon in *Hfx. volcanii* has been shown by RT-PCR.

The metabolic, evolutionary and structural implications of this large multienzyme complex in extremophilic Archaea will be discussed.

Perham, R.N. (1991) Domains, motifs and linkers in 2-oxoacid dehydrogenase multienzyme complexes: a paradigm in the design of a multifunctional protein. *Biochemistry* **30**, 8501-8512.

Jolley, K.A., Maddocks, D.G., Gyles, S.L., Mullan, Z., Tang, S-L., Dyall-Smith M.L., Hough, D.W., Danson, M.J. (2000) 2-Oxoacid dehydrogenase multienzyme complexes in the halophilic Archaea? Gene sequences and protein structural predictions. *Microbiology* **146**, 1061-1069.

Danson, M.J., Morgan, D.J., Jeffries, A.C., Hough, D.W. & Dyall-Smith, M.L. (2004) Multienzyme complexes in the Archaea: predictions from genome sequences. In *"Halophilic Microorganisms" (Ed. A. Ventosa) Springer-Verlag.*

S3A:2

SPECIALIZED ROLES OF THE TWO PATHWAYS FOR THE SYNTHESIS OF MANNOSYLGLYCERATE IN OSMOADAPTATION AND THERMOADAPTATION OF *RHODOTHERMUS MARINUS*

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Rhodothermus marinus accumulates mannosylglycerate (MG) as a major compatible solute in response to growth at supraoptimal temperatures and/or salinity [1]. Two alternative pathways for the synthesis of MG were found in this thermophilic bacterium [2]. The existence of two distinct routes to produce the same final product seems redundant, and leads us to question what might be the physiological significance of such feature. Is this pathway duality related to the dual accumulation behaviour as a response to different environmental aggressions? If so, how is this response regulated, and at what level? To answer these questions, both pathways were genetically and biochemically characterized in detail. In one of the pathways, GDP-mannose is condensed with D-glycerate to produce MG in a single reaction catalyses by mannosylglycerate synthase (MGS), whereas in the other pathway, mannosyl-3-phosphoglycerate synthase (MPGS) catalyses the condensation of 3-PGA with GDP-mannose into a phosphorylated intermediate, mannosyl-3-phosphoglycerate (MPG), which is then hydrolysed to MG by MPG phosphatase (MPGP). It was found that the *mgs* gene (encoding the synthase of the single-step pathway) is not found in the regions flanking the *mpgs* and *mpgp* genes (encoding the enzymes of the two-step pathway), which are organized in an operon-like structure. Both recombinant synthases share most in vitro biochemical and kinetic properties. Surprisingly, the recombinant MPGS shows a low specific activity compared to other homologous MPGSs [3] and contained ca. 30 additional residues at the C-terminus. Truncation of this extension produced a protein with a 10-fold higher specific activity. Moreover, the activity of the complete MPGS was enhanced upon incubation with *R. marinus* cell extracts, and protease inhibitors abolished this activation. Therefore, the C-terminal peptide of MPGS was identified as a regulatory site for short-term control of MG synthesis in *R. marinus*. In addition, the control of gene expression by heat and osmotic stress was studied by immunoassay analysis: the level of MGS was selectively enhanced by heat stress, whereas MPGS was overproduced in response to osmotic stress. We conclude that the two alternative pathways for the synthesis of MG are differently regulated at the level of expression to play specific roles in the adaptation of *R. marinus* to two different types of aggression. This is the only example of pathway multiplicity being rationalized in terms of the need to respond efficiently to distinct environmental stresses.

[1] Silva Z., Borges N., Martins L.O., Wait R., da Costa M.S., Santos H. (1999) Extremophiles 3, 163-172.

[2] Martins L.O., Empadinhas N., Marugg J.D., Miguel C., Ferreira C., da Costa M.S., Santos H. (1999) J. Biol. Chem. 274, 35407-35414.
[3] Empadinhas N., Marugg J.D., Borges N., Santos H., da Costa M.S. (2001) J. Biol. Chem. 276, 43580-43588.

S3A:3

GENOMICS-BASED FUNCTIONAL ANALYSIS OF THE CENTRAL CARBOHYDRATE METABOLISM OF *THERMOPROTEUS TENAX*

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The completed genome sequence of the facultative heterotrophic, hyperthermophilic Crenarchaeon *Thermoproteus tenax* [1] was used in order to predict physiological functions for genes involved in the central carbohydrate metabolism (Emden-Meyerhof-Parnas pathway, Entner-Doudoroff pathway, pentose phosphate pathway, reversible citric acid cycle, glycogen and trehalose metabolism) [2]. This focused approach was chosen in order to combine genomics-based approaches with classical biochemical approaches and thus fill in the gap between sequence and function. Therefore our strategy was to express the genes with predicted function in *Escherichia coli* and to analyze the recombinant proteins for the assigned enzymatic activity. The combination of structural, comparative genomics (e.g. genome organization) with classical biochemical approaches allowed us to i) identify missing links in the central carbohydrate pathways ii) elucidate function for hypothetical proteins and iii) to identify pathways that were supposed to be absent in hyperthermophilic Archaea and even in Archaea. In order to link information about regulation of the central carbohydrate pathways on protein and gene level we have selected this focused approach also for transcriptomics (DNA-microarrays, Northern blot analysis). A summary of our current understanding of the Embden-Meyerhof-Parnas pathway, Entner-Doudoroff pathway [3] and trehalose metabolism will be presented.

[1] Zillig, W., K. O. Stetter, W. Schäfer, D. Janekovic, S. Wunderl, I. Holz, and P. Palm (1981). Thermoproteales: A novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from icelandic solfatares. Zbl. Bakt. Hyg., I. Abt. Orig. C **2**, 205- 227.
[2] Siebers, B., B. Tjaden, K. Michalke, C. Dörr, H. Ahmed, M. Zaparty, P. Gordon, C. W. Sensen, A. Zibat, H.-P. Klenk, S. C. Schuster and R. Hensel (2004). Reconstruction of the central carbohydrate metabolism of *Thermoproteus tenax* by use of genomic and biochemical data. J. Bacteriol. **186**, 2179-2194.
[3] Ahmed, A., Ettema T. J. G., Tjaden, B., Geerling, A. C. M., Van der Oost, J., and B. Siebers (2004). The semi-phosphorylative Entner-Doudoroff pathway in hyperthermophilic Archaea - -a re-evaluation. JBC submitted

S3A:4

STABILIZATION BY CYCLIZATION - RNA AND PROTEIN ENGINEERING EXERCISES

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An important mechanism to control the functionality of biological systems is the regulation of a delicate balance between synthesis and degradation of key components. In the living cell, degradation is brought about by a set of hydrolytic enzymes, each with their specificity to brake the bonds between the building blocks that make up the different types of macro-molecules: carbohydrates, RNAs, and polypeptides. On the basis of the position of the bond that is being attacked, these enzymes can be subdivided into endo- and exo-acting hydrolytic enzymes. Although the continuous turnover of macromolecules has an apparent selective advantage for a cell because it allows for adapting the cell's structural and functional features, the degradation of certain macromolecules (mRNA, protein) can be a serious drawback for potential biotechnological applications. Using molecular engineering exercises on the basis of thermophilic ribozymes and inteins, methods have been developed to generate covalently closed circular molecules of both mRNA and protein in a bacterial host, in an attempt to limit their degradation by exo-acting hydrolytic enzymes.

S3A:5

BIOGENESIS OF SUGAR BINDING PROTEINS IN THE CELL ENVELOPE OF *SULFOLOBUS SOLFATARICUS*

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A large number of secretory proteins in the thermoacidophile *Sulfolobus solfataricus* are synthesized as a precursor with an unusual leader peptide that resembles bacterial type IV prepilin signal sequences. This set of proteins includes the flagellin subunit but also various solute binding proteins. In Gram negative bacteria, type IV prepilin signal sequence containing precursors assemble into large supramolecular structures at the outer envelope of the cell. This involves dedicated assembly and secretion systems. The question arises as to whether *S. solfataricus* binding proteins also assemble into large oligomers, how this is achieved, and what the function of such assembly machines would be. As the cell envelope consists of only a cytoplasmic membrane surrounded by a membrane anchored S-layer, the organization of such complexes might be significantly different from that in Gram-negative bacteria. Here we will report on the functional analysis of the type IV prepilin peptidase and secretion ATPases of *S. solfataricus* that may be involved in secretion and assembly of binding proteins

S3A:6

SPECIAL FEATURES OF THE ATP SYNTHASE ARE REQUIRED FOR OXIDATIVE PHOSPHORYLATION BY ALKALIPHILIC *BACILLUS* AT HIGH PH

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Oxidative phosphorylation (OXPHOS) by extremely alkaliphilic *Bacillus* species represents a major energetic conundrum with respect to the dominant model for energy-coupling in OXPHOS. The Mitchell chemiosmotic model posits a direct relationship between the magnitude of a bulk electrochemical gradient of protons and the capacity for ATP synthesis and also posits that an artificially imposed electrochemical gradient should be as efficacious as one generated by the respiratory chain. OXPHOS by alkaliphilic *Bacillus pseudofirmus* OF4 runs counter to both of these tenets. This organism synthesizes ATP via OXPHOS more robustly at pH 10.5 than at pH 7.5, although the total bulk chemiosmotic driving force is greatly reduced at the higher pH. In addition, an imposed diffusion potential loses its efficacy in energizing ATP synthesis above about pH 9.2 while OXPHOS itself is robust well above that pH (i.e. when respiration itself is generating the driving force). We have hypothesized that special features of the membrane-embedded subunits of the ATP synthase constitute: (i) a pH-dependent gating function that prevents proton loss to the bulk during OXPHOS at high pH and also accounts for the inability of imposed potentials to energize above pH 9.2; and (ii) a mechanism for acquiring protons above the gating pH of 9.2 in a manner that is sequestered from the highly alkaline bulk phase. Six sequence-specific features of the *a*- and *c*-subunits of the *B. pseudofirmus* OF4 ATP synthase have now been changed to the non-alkaliphilic *Bacillus* consensus sequence. All but one of these changes allowed assembly of a functional ATP synthase, as assayed at pH 7.5. The results of a detailed bioenergetic profile strongly support specific roles for the remaining features in OXPHOS at pH 10.5 but not at pH 7.5. Support for a role for three of these features in proton gating at high pH also emerged. Thus adaptations of the OXPHOS machinery itself are required for alkaliphile OXPHOS at high pH in addition to possible global mechanisms for proton retention near the membrane surface.

S3B:1

METABOLIC DIVERSITY OF HALOPHILIC MICROORGANISMS: BIOENERGETIC CONSTRAINTS AFFECTING THE FUNCTIONING OF HYPERSALINE ECOSYSTEMS

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Halophilic and halotolerant microorganisms form a highly diverse group, both phylogenetically and metabolically. However, not all modes of microbial life known in freshwater and marine environments also occur in environments at or approaching salt saturation. Among the dissimilatory processes that do not appear to occur at high salt are autotrophic nitrification, methanogenesis with hydrogen or acetate as energy sources, and acetate oxidation coupled to sulfate reduction. To explain these observations, an analysis was made of the amount of energy generated in the different dissimilatory processes and of the energy cost of osmotic adaptation. The latter varies greatly in accordance with the strategy used to osmotically balance the cytoplasm with the surrounding brines. All microorganisms spend energy to maintain low intracellular sodium concentrations. The *Halobacteriaceae* (*Archaea*) and the anaerobic *Halanaerobiales* (*Bacteria*) use KCl as their intracellular solute, and they have adapted their intracellular machinery to function at high salt concentrations. Other groups synthesize organic osmotic solutes at an even higher energetic cost. By combining information on the energy available to each physiological group and the strategy it uses to cope with salt stress, a model was proposed in 1999 to explain the upper salinity limit at which the different processes occur (Oren, Microbiol. Mol. Biol. Rev. 63: 334). Based on new observations this model can be refined. Growth studies and microcalorimetric measurements have shown that *Halomonas* (an organism that produces ectoine and other organic solutes) grows up to high salinities thanks to a highly efficient carbon and energy metabolism. However, the growth yield of alkaliphilic chemoautotrophic sulfur bacteria decreased with increasing medium salinity. For growth at the highest salt concentrations organic solutes are less suitable. *Salinibacter*, a recently discovered extremely halophilic red prokaryote (*Cytophaga/Flavobacterium* group of the *Bacteria*), resembles *Halobacterium* in its mode of osmotic adaptation. The isolation of a selenate reducing bacterium from the sediments of the Dead Sea that grows at salt concentrations far higher than sulfate reducing bacteria also fits in the general scheme, as selenate reduction is energetically more favorable than sulfate reduction. A coherent model is thus obtained that explains most of the possibilities and limitations of microbial life at high salt concentrations.

S3B:2

NOVEL FAMILIES OF ARCHAEAL VIRUSES FROM HOT ACIDIC SPRINGS IN POZZUOLI, ITALY

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We undertook systematic studies of viral diversity in hot terrestrial environments in Italy. From hot acidic springs (84-91°C and pH 1.5-2.0) in Pozzuoli, seven novel viruses were isolated with double-stranded DNA genomes which infect hyperthermophilic archaea of the genus *Acidianus*. The rod-shaped virus ARV1 was assigned to the family *Rudiviridae* and four different filamentous viruses were classified in the family *Lipothrixviridae*. Two other previously undescribed viruses have unique morphotypes. One, named ABV, has bottle shaped virions with the base densely covered with thick filaments; virions of the other novel virus, named ATV, are pleomorphic and complex particles with an ellipsoid body and tails protruding from each pointed end. These viruses are classified in two new families, *Ampullaviridae* and *Bicaudaviridae*, respectively. ATV

exhibits a property, previously not observed for any virus, that it undergoes a major morphological development outside, and independently of, host cells. The only requirement for the extracellular morphogenesis is a temperature above 75°C. The genomes of all seven new viruses have been sequenced, and the results of sequence analyses will be presented. Analysis of the ATV genome revealed an encoded protein possibly responsible for tail development. Its resembles intermediate filament proteins in its predicted structure and these are known to be involved in the architecture and dynamics of cells.

S3B:3

PHAGE COMMUNITIES IN HOT SPRINGS AND OTHER EXTREME ENVIRONMENTS

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In extreme environments such as hot springs, phage are the only known microbial predators. We have measured prokaryotic and phage community dynamics in these environments. Phage were abundant in hot springs, reaching concentrations of several million viruses per milliliter. Hot spring phage particles were resistant to shifts to lower temperatures, possibly facilitating DNA transfer out of these extreme environments. The phage were actively produced, with a population turnover time of 1-2 days. Phage-mediated microbial mortality was significant, making phage lysis an important component of hot spring microbial food webs. Together, these results show that phage exert an important influence on microbial community structure and energy flow in extreme thermal environments. In addition to community dynamics, we are now measuring phage diversity in hot springs. To do this, DNA from complete hot spring phage communities has been isolate, cloned, and sequenced. These metagenomic analyses show the these phage are very novel and diverse. Currently, these studies are being extended to other extreme environments, including solar salterns and sea ice.

S3B:4

DEEP-SEA PIEZOPHILES, THEIR ECOLOGY, GENOME, AND PHYSIOLOGY

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Piezophilic microorganisms were identified from the deep-sea environment, where have been slaving under high-pressure environment. They could grow better at high-pressure conditions than at atmospheric pressure. To understand the pressure habitat in marine life, several piezophilic bacteria have been isolated from the deep-sea samples, and studied about physiology and molecular genetics for last twenty years. In this year, the genome analysis of two of typical piezophiles, Photobacterium profundum SS9 and Shewanella violacea DSS12, have been performed independently, then the term is coming to the post genome of Piezophiles. In this talk, we would like to review the Piezophiles studies in past, present and future. 1) Piezophiles ecology: Piezophilic bacteria could be isolated from any of deeper ocean, at a depth of more than 2500 m. They are well adapted to deep-sea high-pressure and low-temperature conditions. From the taxonomic study, many of deep-sea piezophiles are closely related with polar psychrophilic bacteria. Therefore the "deep ocean circulation" might be important factor for biological diversity of piezophiles in biogeographic consideration. 2) Piezophiles genome: Japanese group have performed the genome analysis of deep-sea piezophilic bacterium, Shewanella violacea DSS12, which was isolated from Ryukyu-Trench at a depth of 5100 m. From the annotation results, we found many of unidentified genes with the database, so such deep-sea bacterium may have several new genes for adapting such extreme environment. Several useful genes were also identified, and we are expecting to use such genes and products in the biotechnological applications. 3) Piezophiles physiology: At the beginning stage of cell physiology under pressure conditions, several groups had been identified cell divisional problem at higher-pressure conditions. However piezophiles' cells could divide well under such conditions. So we purified the cell divisional protein, FtsZ, from S. violacea DSS12, and confirmed its physical difference with E. coli's one.

S3B:5

MICROBIAL DIVERSITY IN A SOLAR SALTERN

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Solar salterns are ecosystems which contain an increasing gradient of salt until the saturation point of NaCl is reached. The microbial community living under such conditions must be able to adapt to these changes or be replaced by more specialized microbes. During a seven-year period we have been investigating the Cargill solar salt plant in Newark, CA using both cultivation and molecular techniques. Cultivation techniques during that time showed significant changes in the numbers of presumptive Archaea in the higher density pans. Amplicon length heterogeneity fingerprinting of the archaeal community over several years also demonstrated that there were seasonal changes in this community in pans containing 12 to 26% salt. Approximately 600 clones and 100 pure cultures have been sequenced from the same three pans within this salinity range. The samples were obtained in December and June. The results of these studies confirm this seasonal cycling of not only the whole community by both fingerprints and cultivation but also individual genera within the communities of these pans.

S3B:6

SOLAR SYSTEM FOR EXOBIOLOGY: INSTRUMENTATION FOR DETECTION OF EVIDENCE OF EXTRATERRESTRIAL LIFE

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One of the main themes of NASA's present and future goals is the search for extraterrestrial life in our solar system. The Origins theme seeks to search for planets in distant solar systems that may be Earth like and harbor life as we know it or forms not characteristic of our Earth. With regards to Solar System Exploration there are several missions either ongoing or planned for the future to look for evidence of life in our solar system. In the case of Mars, NASA has a separate program to search for evidence of life on Mars and recent probes have detected sub-surface water or permafrost. Liquid water is a basic ingredient that life as we know it must have. Other possibilities are Saturn's moon Titan and Jupiter's icy moon Europa. In both cases, these bodies are believed to have sub-surface oceans where life could exist. Europa is covered with cracks and fissures where liquid water can extrude or be emitted via cryo-volcanism and the same is thought to take place on Titan. What makes Titan very interesting is that it is larger in size than the planet Mercury and has a dense (~ 1.5 bar) nitrogen rich atmosphere similar to that thought to exist for the primordial earth, but very cold with surface temperatures ~ 93 K. The atmosphere also has high concentrations of methane and a zoo of hydrocarbons and nitriles have been detected in its atmosphere. Its surface could be covered with lakes of ethane and a tar like surface which could harbor life or its prebiotic form during random events (i.e., cryo-volcanism, meteoritic impacts) that could temporarily heat its surface to produce liquid water and thus life. Since oxygen is virtually absent in its atmosphere the oxygen in amino acids could be replaced with the "ammono" analog NH. The technology for detecting life in our solar system has been progressing along two separate tracks. One is more along the line of low power (~ mwatts) and low mass (~ 10-100 grams or less) such that miniaturization is encouraged, for which MEMS technology and nanotechnology techniques are being developed. The other track follows along that being advocated by the JIMO/Prometheus mission to Jupiter's icy moons, which will use a fission reactor and provide ~ 100 KW of power and ~ 500 kg of mass to the scientific instruments. We will discuss the numerous kinds of technologies being developed to allow the detection of life in our solar system for a wide range of environmental conditions.

PA:2

FROM GIANTS TO DWARFS: PERSPECTIVES ON THE DISCOVERY OF HYPERTHERMOPHILES

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Most microbial life forms known are mesophiles adapted to ambient temperatures within a range from 15 to 45°C, well corresponding to the temperature of the human habitat. In contrast, communities of super-heatloving ("hyperthermophilic") Archaea (and a few Bacteria) have been isolated which grow optimally (fastest) at temperatures between 80 and 106°C where mesophiles are killed within seconds (e.g. in the Pasteurization process). On Earth, hyperthermophiles are found in water-containing terrestrial and submarine environments of active volcanism where they represent life at the upper temperature border. In addition, hyperthermophiles have been discovered in geothermally heated subterranean oil reservoirs, some 3000 to 4000 meters below the surface. Members of the (non-spore-forming) archaeal genera *Pyrodictium* and *Pyrolobus* were found to survive autoclaving at 121°C, a kind of cosmic impact scenario. As a rule, hyperthermophiles are unable to grow below 60°C (*Pyrolobus*: below 90°C). They are adapted to distinct environmental factors including the composition of minerals and gasses, pH, redox potential, salinity and temperature. A great deal of hyperthermophiles depends only on inorganic nutrients ("chemolithoautotrophic"): inorganic redox reactions serve as energy sources and CO_2 is the only carbon source required to build up organic cell material. The energy-yielding reactions represent anaerobic and aerobic types of respiration. Within Woese's small subunit rRNA-based phylogenetic tree of life, hyperthermophiles occupy all the short deep branches closest to the root. In line with their great phylogenetic diversity, hyperthermophilic archaea display a variety of different unusual cell morphologies like disks, lobes, networks, "golf clubs", giant spheres (15 µm Ø) and giant rods (100µm long). Recently, *Nanoarchaeum equitans* had been discovered, a member of the *Nanoarchaeota*, a so far unknown kingdom of archaea. Cells consist of minicocci, so tiny that they can hardly be recognized under the light microscope (0.4 µm in diameter). Cultivation of *N. equitans* requires the presence of *Ignicoccus sp.*, a coccoid crenarchaeal host. The small subunit rRNA gene sequence remained undetectable in commonly used ecological studies based on the polymerase chainreaction. The *N. equitans* genome is the smallest genome known to date (490,885 bp) and exhibits a limited biosynthetic and catabolic capacity indicating a symbiotic lifestyle. First ecological studies reveal a world-wide distribution of the *Nanoarchaeota*.

S4A:1

ENVISIONING DYNAMIC MOLECULAR MACHINES ACTING IN MAINTENACE OF THE GENOME

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My groups at Scripps and the Lawrence Berkeley National Lab are using systems from extremely thermophilic archaea to help understand the effects of stress on genome maintenance at the molecular level. Our characterizations of the dynamic assemblies from extreme thermophiles integrate DNA repair biology with structure at escalating levels of complexity from domains to multi-protein molecular machines. DNA genetic integrity and cancer avoidance depends upon the structure-specific repair and replication nuclease Flap EndoNuclease (FEN-1) and the processivity factor PCNA. *Archaeoglobus fulgidus* FEN-1 and PCNA complex structures provide a coherent model for DNA substrate recognition and PCNA activation of FEN-1. Together our structural and mutational results support an interface exchange hypothesis for coordinated transfer of DNA intermediates during PCNA-mediated processes. To provide a framework for understanding DNA break repair and recombination machinery, we are similarly defining Pyrococcus furiosus RAD51 interactions with DNA and partner proteins along with those of Rad50 plus the Mre11 nuculease. However, many dynamic molecular machines in humans do not exist in unicellular thermophilic organisms; such as those involved in linking DNA repair to cell cycle, transcription, and replication. Therefore, we are developing a novel eukaryotic system by investigating sequence cDNA from Alvinella pompejana (the Pompei worm), the most thermophilic eukaroyote, as a Rosetta Stone for understanding dynamic molecular machines required for complex coordination in eukaryotic enzyme pathways. These small worms live in tubes mounted on black smoker chimneys where they experience water temperatures measured as high as 80° C on the posterior side of the worm to 20° C on the anterior side (see www.ocean.udel.edu/extreme2002). Thus, A. pompejana are not only experiencing the highest known environmental temperatures for eukaryotes, but also the most eurythermal (temperature gradient). Furthermore, this unique organism is bathed in a toxic soup of heavy atoms and very low pH. Together with Dr. Craig Cary of the University of Delaware, we have successfully harvested A. pompejana samples, collected stabilized nucleic acid samples, and defined sequences from cDNA libraries. The sequences obtained to date have allowed us to clone and structurally characterize the worm's superoxide dismutase (SOD), which protects against reactive oxygen species. The A. pompejana SOD structure sets itself apart from the other structures, in that the crystals diffracted to a higher resolution (1.03 Å) than any other SOD structure. We believe that A. pompejana sequences will provide powerful tools to help researchers address the experimental challenges emerging from interpretations of dynamic protein complexes characteristic of molecular machines that respond to environmental and endogenous stress.

S4A:2

FUNCTION, STRUCTURE AND EVOLUTION OF THE REPLICATION PROTEIN FROM THE ARCHAEAL PLASMID PRN1

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The cryptic plasmid pRN1 from the thermoacidophilic archaeon *Sulfolobus islandicus* encodes a 105 kDa large replication protein. We could show that this 904 amino acid long protein is a novel multifunctional enzyme with ATPase, primase and DNA-polymerase activity[1]. Deletion mutants mapped the primase and DNA-polymerase activity to the N-terminus of the protein, which does not have sequence similarity to known proteins. Point mutants within the N-terminal domain, which we termed prim/pol domain, allowed us to identify three acidic amino acids that are critical for enzyme catalysis and could be involved in the metal ion dependant catalysis of DNA polymerization. The crystal structure of the 200 amino acid long prim/pol domain shows a central depression around the active site lined with basic residues that could be involved in DNA and nucleotide binding[2]. Unexpectedly the prim/pol domain has structural resemblance with archaeal primases suggesting that the replication protein from pRN1 and the archaeal/eukaryotic primases have a common ancestor, which could have been a bifunctional replicase at the advent of DNA replication.

S4A:3

MOLECULAR MECHANISMS OF PROGRESSION AND REPAIR OF REPLICATION FORK IN ARCHAEA

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DNA polymerase requires two protein factors, sliding clamps and clamp loaders, for the processive synthesis of genomic DNA. In archaea and eukarya, proliferating cell nuclear antigen (PCNA), the ring-shaped sliding clamp, encircles double-stranded DNA within its central hole and tether the DNA polymerases onto DNA. Replication factor C (RFC) acts as the clamp loader, which correctly installs the sliding clamp onto DNA strands in an ATP-dependent manner. We succeeded in reconstituting the clamp-loading complex (RFC/PCNA/DNA) using proteins from P. furiosus in the presence of a non-hydrolysable ATP analog. The 3D structure of the complex determined by single-particle electron microscopy reveals two components, a closed ring and a horseshoe-shaped element, which correspond to PCNA and RFC, respectively. The atomic structure of PCNA is well fitted into the closed ring, suggesting that this ternary complex represents a state just after closing of the PCNA ring to encircle a DNA duplex (1). We propose a new molecular mechanism of the clamp-loading, in which the clamp-loading can be completed by ATP binding and some movement of RFC on the PCNA after closing the PCNA ring may provide some spaces for the DNA polymerase to access to the interacting surface of PCNA.

Some efficient repair systems must work to restore the stalled fork for smooth progression of the DNA replication. We found an endonuclease that specifically cleaves nicked, flapped, and fork-structured DNAs in P. furiosus. The enzyme, designated Hef, is well conserved in Archaea (2, 3). This enzyme contains two distinct domains. The analyses of the truncated mutant proteins consisting of each domain revealed that the C-terminal nuclease domain independently recognized and incised fork-structured DNA. On the other hand, the N-terminal helicase domain specifically unwound fork-structured DNA and Holliday junction DNA in the presence of ATP. Moreover, the endonuclease activity of full-length Hef protein was clearly stimulated by ATP hydrolysis. These results suggest that Hef efficiently resolves stalled replication forks by two steps, migrating the branch point to the 5'-end of the nascent lagging strand by N-terminal helicase activbity and following incision of the template strand for leading synthesis by C-terminal nuclease activity. We will discuss stalled replication fork repair in Archaea by our analyses of the proteins RadA, RadB, Hjc, Hjm, and PCNA, in addition to Hef protein.

1. Miyata, T., Oyama, T., Mayanagi, K., Ishino, S., Ishino, Y., and Morikawa, K. (2004) Nature Struct. Mol. Biol. in press
2. Komori, K. Fujikane, R., Shinagawa, H., Ishino, Y. (2002) Genes Genet. Syst. 77, 227-241.
3. Nishino, T., Komori, K., Ishino, Y., and Morikawa, K. (2003) Structure 11, 445-457

S4A:4

THE METHANOTHERMOBACTER THERMAUTOTROPHICUS MCM HELICASE

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DNA polymerases, which replicate chromosomal DNA during the S phase of the cell cycle, require single-stranded DNA as the template, necessitating the separation of the DNA strands. This task is handled by DNA helicases, a group of enzymes that utilize the energy derived from nucleoside triphosphate hydrolysis to unwind the duplex DNA. To date, most of the replicative DNA helicases, those that function at replication forks, were shown to form hexameric rings which translocate along one strand of the duplex and displace the complementary strand. The minichromosome maintenance (MCM) proteins are thought to function as the replicative helicases in archaea and eukarya. The structure of MCM from the archaeon Methanothermobacter thermautotrophicus is currently unclear. In solution the protein appears to form dodecamers. The dodecameric structure is supported by the crystal structure of the N-terminal portion of the molecule. However, electron microscope reconstruction studies of the full-length protein revealed hexameric or heptameric structures, but no larger multimers. Our recent approach to determine the domain(s) required for multimer formation and our attempt to elucidate the structure of the complex will be discussed.

S4A:5

THE DNA REPLICATION MACHINERY OF THE HYPERTHERMOPHILIC ARCHAEON *SULFOLOBUS SOLFATRICUS*

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The complexity of the eukaryotic DNA replication apparatus presents a considerable technical challenge to biochemical analysis of the underlying molecular transactions in this process. In recent years it has become apparent that the DNA replication apparatus of archaea is fundamentally related to but much simpler than that of eukaryotes and as such presents itself as a simplified model to elucidate the core set of interactions and events at the heart of the replication machinery. To this end, we are studying the DNA replication apparatus of the crenarchaeote *Sulfolobus solfatricus* P2. Remarkably, and in contrast to previously characterized archaea and bacteria, *S. solfataricus* possesses multiple origins of replication in its single circular chromosome. The molecular characterization of these origins, their regulation and interaction with archaeal Orc1/Cdc6 homologs will be described. In addition, we have been studying structure/ function relationships in the archaeal MCM complex. Using a combination of mutagenesis and fluorescence resonance energy transfer assays, we have established a model for DNA binding and helicase activities of the MCM complex.

S4A:6

GENETIC IDENTIFICATION OF ARCHAEAL DNA REPLICATION ORIGINS FROM HALOFERAX VOLCANII

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In silico and biochemical studies have shown that the hyperthermophilic archaeon Pyrococcus abyssi initiates DNA replication from a single bidirectional origin, whereas the chromosome of Sulfolobus solfataricus carries three distinct replication origins. To address how Haloferax volcanii replicates its DNA, we report a genetic approach for isolating autonomously replicating sequences (ARS) of this halophilic archaeon. We have constructed genomic libraries in plasmid vectors, which were used to transform H. volcanii. Clones were selected with inserts that supported DNA replication. All clones carried the same genomic region, presumably corresponding to a replication origin. The minimal ARS activity is contained on a 900 bp intergenic sequence upstream of a cdc6/orc1 initiator gene. This sequence contains multiple repeats, albeit different from those of the P. abyssi oriC, and an A/T-rich duplex unwinding element. Pulsed field gel analyses indicate that this sequence is located on the pHV4 megaplasmid (0.7 Mb). Since this replicon is essential and carries a chromosomal-like replication origin, we propose it should be considered as a minichromosome. An attempt to delete this ARS was successful, and resulted in viable

clones with a reduced copy number of pHV4. New ARS libraries were obtained using this deletion strain and allowed the identification of another replication origin, this time situated on the main chromosome (3 Mb). Replication initiation point (RIP) mapping experiments are currently under way to confirm whether these two ARS element function as chromosomal replication origins, and to determine the exact position of the transition between continuous (leading strand) and discontinuous (lagging strand) DNA synthesis. First results indicate that these sequences behave as replication origins in vivo.

S4B:1

THE EXPANDING UNIVERSE OF SMALL NON CODING RNAS IN THE HYPERTHERMOPHILIC ARCHAEON, *SULFOLOBUS SOLFATARICUS*

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In recent years it has become increasingly apparent that non-protein coding RNAs (ncRNAs) constitute a large proportion of the transcriptional output from the genome of an organism. Many ncRNAs genes have been identified in both eukaryotic and archaeal species using computational and biochemical approaches. The majority of these newly identified ncRNAs proved to be previously uncharacterized members of the C/D and H/ACA box RNA families. These small RNAs (sRNAs) associate with specific proteins to form sRNP complexes and participate in the processing and chemical modifications of ribosomal RNA. The archaeal C/D box complex functions as an RNP machine to guide ribose methylation to selected positions in rRNA and tRNA, and contains 3 conserved proteins: aFIB, aNOP56 and L7Ae. The *in vitro* assembly and activity of this RNP methylation machine, reconstituted from purified recombinant components, will be described. The core protein in this complex is L7Ae; This protein, initially annotated as a LSU ribosomal protein, binds to a RNA motif known as the K-turn and plays a central role early in the assembly pathway of the box C/D methylation machine. The K-turn motif is a ubiquitous feature in many RNAs; the L7Ae protein is expected to bind these motifs and play a crutial role in the function of the RNAs. To establish the diversity of L7Ae-associated RNAs, we generated polyclonal antibodies against the L7Ae protein from the archaeon *S. solfataricus*. The antibodies were used in immunoprecipitation experiments to obtain a library of cDNAs specifically associated with the L7Ae protein. Characterization of library entries has resulted in the identification of 34 novel ncRNAs. Of these, only six displayed canonical features of archaeal methylation guide sRNAs, another four contained degenerated C and D box features and one exhibited the structural features common to archaeal H/ACA sRNAs. The remaining 23 sequences lacked the canonical features of C/D box or H/ACA box elements although a K-turn motif could be identified in most of these cDNA clones. Gel retardation assays showed that most of the RNAs recovered from the library, directly bind L7Ae protein. The structure and function of several of these RNAs will be described.

S4B:2

OXIDATION OF INORGANIC SULFUR COMPOUNDS IN ACIDIANUS AMBIVALENS

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The current knowledge of the mechanisms of aerobic oxidation of elemental sulfur to sulfuric acid in the thermoacidophilic archaeon Acidianus ambivalens is summarized focusing on the properties of one soluble and two membrane-bound enzymes. The soluble sulfur oxygenase reductase (SOR) catalyzes the initial step of S oxidation by performing an oxygen-dependent sulfur disproportionation reaction with sulfite, thiosulfate and hydrogen sulfide as products. Reversible denaturation experiments and X-ray crystallography showed that it is made of dimer building blocks, which assemble to a hollow ferritin-like sphere of 24 subunits. The colorless protein contains a low-potential mononuclear non-heme Fe site [Urich, T, et al., 2004, Biochem J, in press] forming a 2-His-1-carboxylate facial triade buried in the interior of each subunit [Urich, T, Frazao, C, et al, 2004, unpublished]. This forms the active site together with 3 cysteine residues. Narrow channels leading to the Fe site suggest that the actual substrate is a soluble sulfur species. The reaction mechanism of the enzyme will be discussed in the light of the X-ray structure and the results of site-directed mutagenesis experiments. The products of the SOR reaction are the substrates of two membrane-bound enzymes, a sulfite:acceptor oxidoreductase (SAOR) and a thiosulfate:quinone oxidoreductase (TQO). The SAOR was purified and consists of 2-3 subunits whose properties are still under investigation [Muller, FH, Bandeiras, TM, et al, unpublished]. The quinone-containing, metal-free and tetrathionate-forming TQO is a 102 kDa glycoprotein of a novel type [Muller, FH, Bandeiras, TM, et al, 2004, Mol Microbiol, in press]. It was composed of two subunits with apparent molecular masses of 28 and 16 kDa, which were previously thought to be part of the terminal oxidase. It oxidized TS with decyl ubiquinone and other artificial electron acceptors at pH 5 in a broad temperature range with an optimum above 90 C. Thiosulfate-dependent oxygen consumption measured in membrane fractions showed that both enzymes can form a short electron transport chain. Models of S oxidation in A. ambivalens will be discussed emphasizing the observation that S is obviously transported into the cytoplasm for the initial oxidation step.

S4B:3

STRUCTURE ANALYSIS OF THE HEAT SHOCK REGULATOR FROM *PYROCOCCUS FURIOSUS*

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We have recently determined the 3D structure of an archaeal regulator of the heat shock response, the heat shock regulator from *Pyrococcus furiosus*, Phr, [1]. Phr is a transcriptional repressor of two heat shock genes (*hsp20* and *aaa+ atpase*) and of its own gene. Promoter bound Phr blocks the binding of RNA polymerase to the TBP/TFB-promoter complex and thereby prevents transcription. Phr is conserved among Euryarchaeota. It shows no sequence similarity to eukaryotic or bacterial regulators with exception of its helix-turn-helix motif, presumably involved in DNA binding. The protein was crystallized with a dimer in the asymmetric unit of the cell. MAD data were collected at the EMBL beamline BW7A at DESY, Hamburg, Germany. The crystal structure was determined by the multiple wavelength anomalous dispersion method (MAD) using a seleno-methionine derivative with 10 selenium sites per dimer and was subsequently refined at 3 Å resolution. Structure analysis revealed a novel folding type among dimeric transcriptional factors, with the following features; (*a*), The N-terminal domain of Phr resembles a helix-turn-helix (HTH) winged-helix DNA binding motif; (*b*), The protein dimerizes with a long anti-parallel 2-helix bundle and a heterogeneous ?-sheet at the central part of the dimer; and (*c*), The C-terminal domain with a strongly negative electrostatic surface potential presumably represents a binding surface for effector molecules. For the identification of the aminoacids relevant for DNA-binding, several mutations of Phr were made. We tested a number of point-mutants with respect to their binding affinities. Mutation of Ser32 in helix 3, for example, had a great influence on binding affinity of Phr to the DNA-promoter sequence. A preliminary DNA binding model will be discussed. To get further insights in the mode of Phr binding, we are currently on the way to crystallize the protein-DNA complex.

Vierke, G; Engelmann, A; Hebbeln, C; Thomm, M, J. Biol. Chem. 278, 18 (2003)

S4B:4

ARCHAEAL PROTEIN TRANSLOCATION SYSTEMS

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Archaea have been identified in a wide range of environments and thus provide a large variety of biologically active molecules. The recent explosion of the number of companies and research institutions spending significant research moneys on the discovery and development of proteins and other bioactive molecules from archaea and "extreme" bacteria is a testament to their great promise in biotechnological and medical advancements. Many proteins of interest are extracytoplasmic and, those that are not, are often genetically engineered to be secreted into the supernatant of their host growth medium in order to simplify their purification. However, heterologous production of archaeal proteins in existing bacterial and eukaryotic expression hosts can be significantly decreased due to inefficient expression and secretion. Thus, understanding the mechanism by which proteins cross the cytoplasmic membranes of archaea is vital for the efficient secretion of native and heterologous proteins. Our lab uses the haloarchaeon *Haloferax volcanii* as the model archeal organism to study the Sec pathway, which secretes unfolded proteins. Using biochemical, genetic, and genomic approaches, we have characterized archaeal homologs of various Sec components. We have shown that haloarchaeal homologs of the universally conserved SRP54 and SRP-receptor, which are vital for co-translational protein translocation in bacteria and eukaryotes, are essential for *H. volcanii* growth. Interestingly, *H. volcanii* homologs of the bacterial SecD and SecF components, which have been suggested to play a role in bacterial post-translational protein translocation, also appear to be crucial, yet not essential, for haloarchaeal growth. A *H. volcanii secDF* knockout strain, while not essential for growth at 45°C, confers a cold-sensitive phenotype, similar to that of an *E. coli secDF* knockout strain. Thus, archaea may use both, the co- and post-translational protein translocation mechanisms to secrete proteins *via* the Sec pathway. These, and other *in vivo* analyses of the haloarchaeal Sec pathway, show that *H. volcanii* is a good model for studying archaeal Sec transport. However, genomic and extensive *in vivo* data from our lab strongly suggest that haloarchaea secrete most of their proteins in a folded conformation via the Tat pathway, a phenomenon that may reflect an adaptation to the high salt conditions they inhabit. Thus, while the haloarchaea have provided us with a very useful model system to study archaeal protein translocation, it also allowed us to identify a novel strategy these organisms use to adapt to an extreme environment.

(More detailed description of the *in vivo* characterization of the *H. volcanii* Tat pathway can be found in the abstract submitted by Kieran Dilks).

S4B:5

CRYO-ELECTRON-TOMOGRAPHY OF THERMOTOGA MARITIMA: CHEMORECEPTOR CLUSTER AND THE FLAGELLAR MOTOR

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Cryo-electron tomography has unique potential to study the structure of intact prokaryotic cells in a close-to-life-state at molecular resolution (4-5 nm) in three dimensions (3-D) [1, 2]. Using automated data acquisition schemes the electron dose can be kept low enough to study ice-embedded biological samples without apparent radiation damage in the electron microscope. Once a 3-D data set (tomogram) is recorded and processed it can be sectioned in silico in any direction desired to display and analyse structural details of the cell's interior. Rendering techniques allow us to visualize these structural features in 3-D. Here, we applied cryo-

electron tomography to the bacterium Thermotoga maritima to get insights into its supramolecular architecture. Whole cells were embedded in vitreous ice by plunge freezing. Single-axis tilt series were recorded under low dose and cryo conditions using the Philips CM300 FEG transmission electron microscope (TEM) operated at 300kV and additionally equipped with a Gatan imaging filter (GIF). The tilt series were recorded typically in an angular range of -60Â° to +60Â° with an increment of 1.5Â°. The resulting 2-D projections were used to calculate a 3-D reconstruction by weighted backprojection. The focus of our work was the structural components of the chemotactic system of Thermotoga maritima. These organisms as well as others are able to sense and to respond to their surrounding environment via the interplay of chemoreceptors, methyl-accepting proteins (MCPs), CheA (a kinase), CheW (linking protein), and CheY that controls the rotation of the flagella. With cryo-electron tomography we studied the major components of this system in situ. In particular we identified the cluster of chemoreceptors near the cell pole, characterized the flagellar motor exhibiting an uncommon structure of the basal body, and we detected a hitherto unknown tunnel-like structure in the cytoplasm that possibly plays a role linking these components functionally.
[1] Plitzko, J.M. et al. (2002) In vivo veritas: electron cryotomography of cells. Trends Biotechnol. 20: S40-S44
[2] Baumeister, W. (2002) Electron tomography: towards visualizing the molecular organization of the cytoplasm. Curr. Opin. Struct. Biol. 12: 679-684

S4B:6

MOLECULES AND STRUCTURES INVOLVED IN THE CELL-CELL INTERACTION BETWEEN *IGNICOCCUS* AND *NANOARCHAEUM*

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The hyperthermophile *Nanoarchaeum equitans* is the first known member representing the novel archaeal kingdom Nanoarchaeota (1,2). *N.equitans* can only grow in coculture with cells of the chemolithoautotrophic, hyperthermophilic archaeum *Ignicoccus* sp. KIN4I. Previous studies have unravelled the unusual ultrastructure of *Ignicoccus* cells: they lack any stable cell wall polymer. Instead, they are surrounded by a huge periplasm, filled with membrane-bound vesicles, and by an outer membrane, structural features not known for any other archaeon (3). *N.equitans* cells are the smallest cocci known today, with a diameter of about 400 nm and a cell volume of only 1/100 of that of *E.coli*; they have the smallest archaeal genome sequenced to date, and also one of the most compact; they lack genes for synthesizing lipids, cofactors, amino acids, or nucleotides (4). Growth of *N.equitans* is strictly dependent of direct and tight contact with *Ignicoccus* cells. Therefore, we must assume that the interaction is highly specific and that specialised transporter molecules are involved. In order to understand the symbiotic relationship between these two archaea, we currently characterize the surface molecules, which are involved in this interaction, and analyse their ultrastructure, using 3D electron microscopy. So far, we have found that the lipids in both microorganisms are derivatives of caldarchaeol; the lipid composition in both organisms is very similar (Jahn et al., in preparation). The outer membrane of *Ignicoccus* contains several pore-forming proteins, which might be used for specific or unspecific transport processes (5). The surface of *N.equitans* cells is covered by multiple copies of a glycoprotein, forming an S-layer with p6 symmetry and a center-to-center distance of 15 nm (2). The sequence of the S-layer protein and its 3D structure, as determined by electron crystallography, is unique among archaeal S-layers. Together with its rDNA sequence and the sequences of several proteins, this confirms the unique phylogenetic position of *N.equitans*. At present, we aim to analyse the structure and function of the outer membrane proteins of *Ignicoccus*, and to visualise the contact site by electron tomography. Lit: (1) Huber H et al. 2002. Nature 417:63-67 (2) Huber H et al. 2002. Res. in Microbiol. 154:165-171 (3) Rachel R et al. 2002. Archaea 1:9-18 (4) Waters E et al. 2003. PNAS 100:12984-12988 (5) Naether D and Rachel R. 2004. Biochem.Soc.Trans. 32:199-203

S5A:1

THE GENOME SEQUENCE OF THE THERMOACIDOPHILIC ARCHAEON PICROPHILUS TORRIDUS AND ITS IMPLICATIONS FOR METABOLIC AND MOLECULAR ADAPTATION TO LIFE AROUND PH 0

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The euryarchaeal species of the genus Picrophilus, P. torridus and P. oshimae, are able to grow at pH values around pH 0 at growth temperatures up to 65°C, thus they represent the most thermoacidophilic organisms known. The high specialization of P. torridus for growth in extremely hot and acidic habitats is evident from its inability to grow at pH values above 4.0 and its low intracellular pH (4.6), and makes it a model organism to study thermoacidophilic adaptation. P. torridus is an obligately aerobic heterotroph which probably thrives as a scavenger. Sequence analysis of the P. torridus genome yielded a 1.55 Mb large single circular chromosome wich encodes 1,536 proteins. To our knowledge, P. torridus has the smallest genome among aerobic, non-pathogenic microorganisms growing on organic substrates. The following features deduced from comparative genome analysis may contribute to the thermoacidophilic survival strategy of P. torridus: (i) The small genome of P. torridus with the highest coding density among thermoacidophiles indicates that genome reduction may have been important for the evolution of adaptation to the combination of extremely low pH and high temperature. (ii) Comparison of the amino acid composition of P. torridus proteins with values deduced from other genomes suggests that an increased (surface) hydrophobicity may contribute to their acid tolerance. (iii) An exceptionally high ratio of secondary over primary ATP-consuming transporters demonstrates that the high proton concentration in the surrounding medium is extensively used for solute transport. (iv) Finally, it appears that certain genes which may be particularly supportive for

the extreme lifestyle of P. torridus have been internalized into the genome of the Picrophilus lineage by horizontal gene transfer from the phylogenetically distant crenarchaea and bacteriathat horizontal gene transfer from crenarchaea and bacteria. Also, it is noteworthy that thermoacidophiles from phylogenetically distant branches of the Archaea apparently share an unexpectedly large pool of genes, since 66 % of all P. torridus genes were found to have orthologs in the related euryarchaeon Thermoplasma acidophilum but also 58 % in the crenarchaeon Sulfolobus solfataricus.

S5A:2

A SYSTEMS APPROACH TO ELUCIDATE DNA REPAIR MECHANISMS AND REGULATION IN THE ARCHAEA

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Organisms devote a considerable amount of their resources to the repair of DNA damage with a multitude of specialized proteins, and overlapping pathways, designed to cope with all possible insults to their genetic material. In Archaea, little is known about the pathways for DNA repair, and almost nothing is known about their regulation. We used the extremely halophilic archaeon, *Halobacterium sp.* strain NRC-1, as model system for a molecular-level study of cell survival under UV and gamma irradiation. *Halobacterium* sp. is highly resistant to exposure to radiation, with survival without loss of viability up to 110 J/m^2 of UV-C and 1000 Gy of gamma-ray. Using functional genomics, we investigated the proteins and regulatory networks involved in the repair of lesions produced by UV (photoproducts) and ionizing irradiation (oxidative damage and DNA double-strand breaks). Data analyses provided no evidence of a coordinated DNA repair response to DNA damage. This is similar to what we found with the hyperthermophilic archaeon, *Pyrococcus furiosus*, but in contrast to the SOS-repair system found in Bacteria. In addition to photoreactivation, three other putative repair mechanisms were identified including a methylation-directed mismatch repair, four oxidative damage repair enzymes, and two proteases for eliminating damaged proteins. Both gamma and UV irradiation induced down regulation of many important metabolic functions during repair, which seems to be a phenomenon shared by all three domains of life. Finally, we identified several transcriptional regulators and protein kinases that are highly regulated by UV and gamma irradiation.

S5A:3

INSIGHT INTO COLD ADAPTATION IN ARCHAEA FROM GENOMIC AND PROTEOMIC STUDIES

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Temperature is a critical environmental factor controlling the evolution and biodiversity of life on Earth. The majority of the Earth's biosphere is permanently cold. Archaea contribute significantly to biomass in cold environments, although only a handful have been isolated. Underpinned by the genome sequence data for *Methanococcoides burtonii*, we developed proteomics using two dimensional electrophoresis (2DE) and liquid chromatography-mass spectrometry (LC-MS), and have achieved the first global view of the biology of cold-adaptation in *M. burtonii*. Proteins specific to growth at 4°C versus 23°C (T_{opt}) were identified by 2DE, and 43 differentialy expressed proteins were identified by MS. mRNA levels were also determined for all 43 genes. Cold adaptation was linked to changes in fundamental cellular processes including metabolism, transcription and protein folding, with key roles identified for the pyrrolysine-containing trimethylamine methyltransferase, RNA polymerase subunit E, a response-regulator from a two component regulatory system and peptidyl prolyl *cis/trans* isomerase. Increased levels of the heat shock protein, DnaK were observed during growth at 23°C, indicating that growth of cold-adapted organisms at apparently optimum temperatures is stressful. (Goodchild et al, 2004, Molecular Microbiology, 53: 309-321.) Gene annotation and gene organisation from the draft genome was linked to LC-MS analysis of the expressed-proteome to define the key biological processes functioning at 4°C. 528 proteins ranging in p*I* from 3.5 to 13.2, and 3.5 - 230 kDa, were identified. Knowledge of the expressed proteins advances our level of understanding of the biology of the cell from coding potential to actual process, and highlights a mechanistic complexity that must be managed by the cell. For example, the eucaryotic-like, core RNA polymerase machinery is simultaneously interacting with bacterial-like anttiterminator and numerous response regulator proteins to enable transcription. As a second example, transposases are expressed during normal, steady-state-growth and are therefore not just dormant relics of past evolutionary events, but are active and likely to be affecting the cell's genetic composition and structure. The expression of the transposases therefore has major implications, ranging from effects on the genetic diversity and fitness of natural populations through to a previously unrealised value for the development of genetic tools in *M. burtonii*. Characteristics of the fundamental cellular processes inferred from the expressed-proteome highlight the evolutionary and functional complexity existing in this domain of life. The study also demonstrates the capacity to perform high-throughput analyses of proteins from psychrophiles.

S5A:4

COMPARATIVE ANALYSIS OF THERMOTOGA SPECIES

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Initial genome comparisons of *Thermotoga maritima* have revealed a high degree of genetic diversity, and present evidence for lateral gene transfer between members of this lineage and archaeal species. In an attempt to estimate the extent of genomic diversity across the Thermotogales, a comparative genomic hybridization study was initiated to compare 12 *Thermotoga* strains to the sequenced *T. maritima* MSB8. These 9 strains were isolated from different locations throughout the world, and based on phylogeny of the 16S rDNA were proposed to be closely related to each other. The CGH study has revealed many differences across the strains that could be associated with possible substrate utilization patterns, most likely a reflection of the environmental niche that these individual species occupy. Results from the analysis of the genome sequence of the recently completed *T. neapolitana* will also be presented.

S5A:5

STRESS RESPONSES OF PYROCOCCUS FURIOSUS

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Pyrococcus furiosus grows optimally near 100°C using peptides and sugars as carbon and energy sources. It converts them to organic acids, carbon dioxide and hydrogen gas. The organism also reduces elemental sulfur (S°) to hydrogen sulfide. It is routinely grown in 600 liter cultures and from the biomass a variety of enzymes involved in hydrogen metabolism have been characterized. The genome of P. furiosus is 1.9 Mb in size and contains 2,065 annotated open reading frames (ORFs). Like most prokaryotic genomes, approximately 60% of the ORFs show sequence similarity to genes of known function in other organisms, while the remainder is of unknown function. Determining the role of these so-called conserved/hypothetical ORFs is one of the major challenges of the post-genomics era. Whole genome DNA microarray analysis is being used to provide insight into the response of P. furiosus to various environmental stresses. Many of them involve previously uncharacterized hypothetical ORFs, and at least some indication of their function is now apparent. Results will be presented on how P. furiosus responds to a change in carbon source, to oxidative shock and to growth at sub-optimal temperatures. The results of these studies will be discussed with a particular emphasis on the roles of conserved/hypothetical ORFs. To date, at least some indication of biological function has been obtained on more than 100 of the 830 conserved/hypothetical ORFs in P. furiosus.

S5A:6

FUNCTIONAL GENOMICS OF THE MODEL HALOPHILE *HALOBACTERIUM* SP. NRC-1

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The halophilic Archaeon (haloarchaeon), *Halobacterium* sp. NRC-1, is an excellent model for post-genomic analysis. It is easily grown in hypersaline medium containing about a ten-fold concentration of seawater. Its genome is completely sequenced and a large number of genetic tools are available for its characterization. We have targeted several aspects of the fundamental biology of *Halobacterium* NRC-1 for detailed study using functional and comparative genomic approaches. Among these are its aerobic, anaerobic, and phototrophic physiological capabilities, its responses to environmental stresses, and its acquisition of apparently bacterial genes from the environment. *Halobacterium* NRC-1 grows best organotrophically by aerobic respiration or anaerobic fermentation, but it also has the capability for anaerobic respiration and photophosphorylation. The regulation of the physiological switch between these physiological states is determined by novel regulatory genes, including a family of PAS-PAC and GAF domain proteins and other regulators. We have studied a number of these genes by gene knockouts, proteomic analysis, and DNA microarrays. Our results indicate a hierarchy in the physiological states experienced by cells and suggest a complex network of regulators. Evidence for the involvement of specific regulators and transcription factors in transcription of genes has also been obtained. A number of these studies, including those recently published, have shed light on the ability of this extremophile to survive and flourish in an environment containing essentially saturated salts.

S5B:1

PHYSIOLOGICAL AND ECOLOGICAL SIGNIFICANCE OF GLYCOSIDE METABOLISM IN HYPERTHERMOPHILIC HETEROTROPHS

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Given the information encoded in the genomes of heterotrophic hyperthermophiles sequenced to date, it is apparent that glycosides play an important role. The genomes of two well-studied microorganisms in this group, the bacterium Thermotoga maritima and the archaeon Pyrococcus furiosus, encode more carbohydrate active enzymes per Mb of sequence than most other bacteria and archaea for which such information is available. The capability to manipulate glycosides for nutritional or ecological purposes is apparently an important aspect of certain hydrothermal niches. As such, we have employed functional genomics approaches to examine a number of aspects of glycoside metabolism in T. maritima and P. furiosus ranging from nutritional diversity to surface colonization to inter- and intraspecies interactions in high temperature environments. Results of these studies with these two model microorganisms will be presented.

S5B:2

EVIDENCE THAT A PHOSPHOMANNOMUTASE FROM SULFOLOBUS SOLFATARICUS IS REGULATED BY PHOSPHORYLATION

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Analysis of the tryptic peptides from a soluble extract from the extreme acidothermophilic archaeon Sulfolobus solfataricus revealed that serine residue 309 in a putative phosphomannomutase was phosphorylated. The gene encoding this enzyme was cloned and its protein product expressed in E. coli as a recombinant fusion protein. The recombinant protein exhibited phosphomannomutase activity in vitro as predicted. Examination of the X-ray structures of homologous phosphomannomutases suggested that serine-309 resided near the mouth of the active site pocket. If so, a phosphoryl group present at this location might be expected to impair binding of the anionic substrate via an electrosteric mechanism analogous to that by which the isocitrate dehydrogenases from enteric bacteria are regulated by phosphorylation. Thus, phosphorylation would be predicted to inhibit activity. To test this hypothesis, mutagenic alteration was used to replace serine-309 with alanine, glutamine, or glutamate. As expected, the alanine-containing enzyme form exhibited near wild-type activity, while the glutamate-containing form was virtually inactive. The activity of the glutamine-containing form was intermediate between the two, suggesting that the greater size of glutamine vis-a-vis serine may offer some degree of steric obstruction in and of itself. Efforts are currently underway to identify the enzyme responsible for the covalent phosphorylation of serine-309.

S5B:3

NEW EXTREME THERMOPHILES FROM HIGH TEMPERATURE COMPOST

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It is well known that the inside of compost is hot environment due to heat produced by bacterial fermentation. Compost soil was classical source for isolating moderate thermophiles belonging to Geobacillus, Bacillus, and Clostridium. Recently "high temperature" compost have been developed. The internal temperature of high temperature compost reaches up to 95°C or even higher, and this high temperature lasts for a week or so. Recently the author and his colleagues have succeeded to isolate new extreme thermophiles from high temperature compost. A typical strain YMO81 is an aerobic, long rod, and capable of growing at 83oC. Though 16S rDNA base sequence of YMO81 showed considerable similarity to that of *Geobacillus stearothermophilus*, the isolate is non-spore forming and gram negative. Electron microscopic observation confirmed the presence of a typical outer membrane characteristic to gram negative bacteria. Phylogenetic analysis revealed that the new isolates comprise an independent group from Bacillus-Clostridium species. We wish to propose that the isolate belongs to a new genus. Extreme thermophiles often produce unique polyamines and in some cases these unique polyamine(s) is essential for the growth at high temperature extremes. The new isolates produce N^4-3-aminopropylspemine, a very rare polyamine, as the major component. We confirmed the chemical structure by organic synthesis. Roles of unique polyamines in thermophily will be discussed.

S5B:4

THE ROLE OF COENZYME A DISULFIDE REDUCTASE AND SMALL INTRACELLULAR THIOLS IN THE METABOLISM OF PYROCOCCUS

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Studies in this lab have shown that *Pyrococcus* contains an NADH oxidase and a coenzyme A disulfide reductase, two flavoproteins which are proposed to act in the oxidative stress response of this strict anaerobe. We have demonstrated that *Pyrococcus furiosus* contains a high concentration of the coenzyme A thiol, consistent with a role for this thiol in the maintentence of the internal redox environment of this organism. Previous microarray studies (Schut, G. J., Zhou, J., and Adams, M. W. W. (2001) *J. Bacteriol.* 183, 7027-7036) have shown that the CoADR of *P. furiosus* (referred to in that study as *nox A-2*) is upregulated 5-fold during growth with elemental sulfur as a terminal electron accepting agent, suggesting that the coenzyme A thiol/disulfide may be either directly or indirectly involved in the transfer of electrons to sulfur. Current studies are examining the effect of growth in the presence of S^0, growth with different carbon sources, and growth following exposure to the disulfide stress inducing compound diamide on the concentration of small molecular weight thiols in *Pyrococcus*. The Pyrococcal CoADR is quite distinct from the mesophilic CoADR, consistent with different roles and mechanisms for the two enzymes. While it shows a preference for NADPH, the Pyrococcal CoADR is able to efficiently use both NADPH and NADH, while the Staphylococcal version of the enzyme shows a strong preference for NADH. This difference is consistent with both the presence of other unique NADP(H)-dependent enzymes in *Pyrococcus* and the high NADP(H)/NAD(H) ratio of this organism. The Pyrococcal enzyme also stabilizes different intermediates during its reductive and oxidative half reactions, does not show the subunit asymmetry observed with its mesophilic counterpart, and is a tetrameric rather than dimeric enzyme, suggesting that it may utilize different catalytic pathways.

S5B:5

ACTIVITY-STABILITY RELATIONSHIPS IN EXTREMOPHILIC ENZYMES

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The conformational stability, irreversible thermal unfolding, thermodependence of activity, structural permeability and the thermodynamic basis of these processes in á-amylases, xylanases and DNA ligases from psychrophilic, mesophilic and thermophilic microorganisms have been studied. An energy landscape for these extremophilic enzymes, based on the folding funnel model and integrating the main differences in conformational energy, cooperativity of protein unfolding and temperature dependence of activity is proposed. The shape of the base of the funnel, which depicts the stability of the native state ensemble, illustrates the thermodynamic parameters of activation that characterize these extremophilic enzymes, thereby providing a rational basis for stability-activity relationships in proteins adapted to extreme temperatures.

S5B:6

THE FIRST CRYSTAL STRUCTURE OF HYPERTHERMOSTABLE NAD-DEPENDENT GLUTAMATE DEHYDROGENASE FROM *PYROBACULUM ISLANDICUM*

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The investigations on the biochemical properties and primary structures of hyperthermophilic NADP-specific glutamate dehydrogenase (NADP-GluDH, EC 1.4.1.4) have been mainly carried out on those from marine hyperthermophiles such as *Pyrococcus* (*Pc.*) and *Thermococcus* genera to date. Besides NADP-GluDHs, we have found an NAD-specific GluDH (EC 1.4.1.2) in a continental hyperthermophilic archaeon *Pyrobaculum* (*Pb.*) *islandicum*. Comparatively low sequence homology (below 48%) of the enzyme with those of NADP-GluDHs was observed in contrast to high sequence homologies (above 82%) observed among NADP-GluDHs from hyperthermophiles. The NAD-GluDH is extremely thermostable and does not lose the activity by incubation at 100°C like *Pc. furiosus* GluDH. In addition, the enzyme is also highly resistant to denaturants, organic solvents and detergents . We have analyzed 3D-structure of this enzyme for the better molecular understanding. In this paper, we report the structure determination of *Pis*-GluDH and compare with those from hyperthermophile and mesophile enzymes. The homohexameric structure of Pis-GluDH was solved and refined at a resolution of 2.8Å with a crystallographic R-factor of 20.2% (Rfree=26.6%). The structure indicates that each subunit consists of two domains separated by a deep cleft containing an active site. The secondary structural elements and catalytically important residues of the enzyme were highly conserved among NAD(P)-dependent GluDHs from other sources. From the analysis of the 3D structure, hydrophobic interactions between intersubunits were found to be one of important features for the enzyme oligomerization. It has been reported that the *Pc. furiosus* GluDH is highly thermostable similar to the *Pb. islandicum* GluDH and the increase of intersubunit ion-pair networks is responsible to the extreme thermostability. However, the number of intersubunit ion-pairs in the *Pb. islandicum* GluDH molecules was much less than those of the *Pc. furiosus* enzyme. On the other hand, in the case of *Pb. islandicum* GluDH the hydrophobic interaction in the intersubunit was highly strengthened and responsible to the extremely high thermostability. This indicates that the major molecular strategy for high thermostability of GluDHs from different hyperthermophiles is largely different each other even with same enzymes.

S6A:1

ENHANCED EXPRESSION OF A THERMOPHILIC BACTERIAL XYLANASE (XYNB) IN TRICHODERMA REESEI

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Heterologous expression and secretion of a thermophilic bacterial xylanase in *Trichoderma reesei* involves several factors that need addressing. For example, it is critical that the codon usage of the foreign gene to be expressed matches that of the fungal host[1]. Also, the use of an appropriate expression vector equipped with the features for proper processing is essential. These include space for signal peptidase cleavage, a proteolytic Kex-like cleavage site for secondary processing and, when necessary, a pro-fragment from a known secreted protein for proper folding[2,3]. We previously showed that high levels of mRNA were produced from a fusion of a codon-optimised, synthetic *xynB* from the extreme thermophile *Dictyoglomus thermophilum* Rt46.B1 to the CBH1 signal sequence but low levels from a *xynB* fusion to the CBH1 core-linker region[1]. Unfortunately, very little or no XynB product was found to be secreted into the culture medium (1). Thus, three new expression plasmids (pHEN54, pHEN54RQ and pHEN*xyn1pro*RQ), all adopting the CBH1 signal sequence *xynB* fusion strategy but containing the above-mentioned features for proper processing and secretion of the XynB protein, were tested. All three constructs resulted in the thermophilic XynB being secreted into the medium. Enzyme assays showed significant xylanase B enzyme activity in the culture supernatants of several transformants, but the best activities came from the ones transformed with the pHEN54RQ-*xynB* construct. Furthermore, the bacterial xylanase appears to be glycosylated in *T. reesei,* whereas it is not when expressed in bacteria. In this presentation, we discuss the effects of the above-mentioned structural features on the production of a thermostable xylanase from *D. thermophilum* in several *T. reesei* strains.

[1]Te'o, V. S. J., A. E. Cziferszky, P. L. Bergquist and K.M. H. Nevalainen. FEMS Microbiol. Lett, **190**, 13-19 (2000).
[2]de Faria, F.P., V.S.J. Te'o, P.L. Bergquist, M.O. Azevedo and K.M.H. Nevalainen et al.,. Lett. Appl. Microbiol. **34**, 119-123 (2002).
[3]Bergquist, P. L., V. J. S. Te'o, M. D. Gibbs, A. C. E. Cziferszky, F. P. de Faria, M. O. Avezado and K. M. H. Nevalainen. Prog. Biotechnol. **18**, 239-246, (2002).

S6A:2

RE-DESIGNING LIFE *IN-SILICO*

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To sustain life, all living cells must have a constant -supply of energy and matter. It has been found that the strategies and or enzymatic reactions used to transform this energy and matter within the cells, varies to a high degree, depending on the environmental conditions where life has evolved. Since ancient times, we have utilized the metabolic capabilities of microorganisms to obtain a wide range of products. Modern biotechnology has increased our ability to improve microorganisms to obtain better commercial processes. However, one of the problems to optimize cellular metabolism to obtain a particular product, is that each modification that we do, may impact the physiology of the cells. Considering that at any giving time, a cell is performing thousand of enzymatic reactions, the prediction of the outcome of a particular modification to the metabolic pathways of a cell, has been a challenge. We have developed a computer algorithm to calculate the full spectrum of possible phenotypes open to a microorganism in seconds versus hours or even days, as was previously the case. This algorithm coupled to a curetted database that contains more than 3500 enzymatic reactions described in the literature, enable us to redesign cellular physiology and better exploit what Nature has selected through billions of years of evolution. An overview of this approach will be discussed in this resentation.

S6A:3

CHARACTERIZATION OF THERMOPHILIC MICROBIAL CONSORTIA ASSOCIATED WITH OIL

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Oil reservoirs harbour a variety of microorganisms including thermophilic sulfate-reducing bacteria (tSRB), nitrate-reducing bacteria (tNRB) and methanogens. Microbial communities in oil reservoirs play important roles in oil biodegradation and reservoir "souring" (the production of sulfide by SRB). Souring is costly to the oil industry due to associated problems such as accelerated corrosion and plugging, so oil companies aim to inhibit or kill SRB. Sulfate reduction can be inhibited directly via nitrite. Nitrite inhibits sulfate reduction as it is a competitive inhibitor of dissimilatory sulfite reductase the terminal enzyme in the sulfate reduction pathway. Some SRB possess a nitrite reductase which catalyzes nitrite removal, thus enabling them to overcome nitrite inhibition. Although the problem of souring in mesophilic environments has been well investigated, thermophilic environments such as those found in deep oil reservoirs have been less studied. Microorganisms present in two different tSRB enrichments from the North Sea and an oil storage tank consortium have been characterized in this work. The North Sea tSRB enrichment cultured on a combination of acetate, propionate and butyrate was effectively inhibited by 0.5 mM nitrite and the North Sea tSRB enriched on lactate only was inhibited effectively by 0.25 mM nitrite. Sequencing of 16S rDNA from the North Sea tSRB enrichment grown on acetate, propionate and butyrate indicated the presence of *Thermodesulforhabdus norvegicus*. The oil storage tank consortium had tSRB, tNRB, nitrate-reducing sulfide-oxidizing bacteria (NR-SOB) and methanogenic activity; 4 mM nitrite was required to completely inhibit sulfate reduction. Isolate,tSRB-8A was inhibited by 0.5 mM nitrite. This consortium was able to anaerobically degrade oil organics, volatile fatty acids (VFA), hexadecane or acetate to produce methane. *Garciaella nitratireducens*, three *Clostridium spp.,* and a strain of *Methanothrix thermophila* were identified by 16S rDNA sequencing. Based on a comparison with the literature the storage tank consortium appears to contain oil-degrading bacteria that produce organic acids and hydrogen, which feed the SRB and NRB. Understanding their interactions will allow more effective control of souring in the future.

S6A:4

BIOLOGICAL NEUTRALIZATION OF HIGHLY ALKALINE TEXTILE WASTE WATER USING AN EXTREMOPHILE, *EXIGUOBACTERIUM SP.* (DSM ID 03-501)

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In the endeavor of making the environment pollution-free, some industries are enforced to treat their waste water before its release in the environment. Waste waters emanated from industries may be toxic, hot, highly acidic or alkaline, colored etc. where general micro-flora can not exist. Textile mill effluent is such type of waste water which is highly alkaline (pH-13) and hot (50-60^{0}C) and thus it is difficult to treat because general micro-flora can not survive in such adverse conditions. Present study exploits the capability of an extremophile, *Exiguobacterium sp.* (DSM ID 03-501) which was isolated from the alkaline soil near to textile mill. This bacterium is a gram positive rod and partial 16S rDNA sequence shows a similarty of 99.4% to *Exiguobacterium aurantiacum*strain ? Z8? but the physiological results are different which show its novelty. This bacterium has been found capable to grow in a medium of pH 12.2 and capable to neutralize textile industrial waste water from pH 12.2 to pH 7.5 within two hours. Alkaline bacillus medium (ABM) was selected as the suitable medium and maximum growth of one liter culture could be achieved in eight hours when incubated at 45^{0}C/ 200 rpm. For the neutralization of textile waste water, eight hours grown culture was centrifuged and the pellet was added to the hot textile waste water(55^{0}C) of pH 12.2. Lowering of pH from 12.2 to 7.5 using this bacterium could be achieved in a period of two hours . Enzyme assays for xylanase, cellulase and amylase were also found positive. Pilot scale study of biological neutralization using this extremophile has started at plant site.

S6A:5

PRESSURE PERTURBATION CALORIMETRIC STUDIES OF BIPOLAR TETRAETHER LIPOSOMES DERIVED FROM THE THERMOACIDOPHILIC ARCHAEON *SULFOLOBUS ACIDOCALDARIUS*

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The polar lipid fraction E (PLFE) is a mixture of bipolar tetraether lipids, existent as a major lipid component in the thermoacidophilic archaeon *Sulfolobus acidocaldarius*. PLFE liposomes exhibited remarkable stability against temperature, pH, and the actions of hydrolytic enzymes and were used as a model for studying archaeal plasma membrane and for developing technological applications. In this study, pressure perturbation calorimetry has been used to further characterize the membrane packing in PLFE liposomes. For PLFE liposomes derived from cells grown at 78°C, the plot of the thermal volume expansion coefficient, a, (pH 2.1) versus temperature, T, exhibits two peaks centered at ~43-45°C and 58-60°C, in agreement with those observed by small angle X-ray diffraction. The a-vs-T plot yields the relative volume change, DV/V, of 0.10-0.14% and 0.08-0.09% for these two transitions. These values are smaller than the DV/V of the main phase transition (2.8%) and the pre-transition (0.2%) of the monopolar diester lipid dimyristoylphosphatidylcholine. The low DV/V echoes that PLFE liposomes are rigid, tightly packed, and thermally stable. For PLFE liposomes derived from cells grown at 65°C, the temperature variation of a shows only one transition (~43°C) with a higher DV/V value (0.56%, pH 7.0). The increase in DV/V with decreasing cell growth temperature can be attributed to the decrease in the number of cyclopentane ring in PLFE. Alternatively, the less tight packing inferred by a higher DV/V may result from an increase in the negative charge on the phosphatidylinositol moiety of PLFE due to an increase in pH from 2.1 to 7.0. An increase in DV/V during the transition should increase solute permeation across PLFE liposomal membranes. These results may be useful for designing archaeosomal drugs or vaccines.

S6A:6

DENATURATION OF AN EXTREMELY STABLE TETRAMERIC HYPERTHERMOPHILIC PROTEIN

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Proteins from extremophiles have potential applications as biocatalysts in industrial processes at extremes of temperature, pressure, and harsh chemical conditions. Expression of extremophilic proteins in both native and recombinant systems yield only moderate amounts of protein. Recognizing unique determinants of stability to engineer higher stability through single mutations has also proven to be elusive. By systematically studying the stability relationships between extremophilies and their mesophilic homologues, determinants of stability dependent on specific folds or functions could be determined. To this end, we seek to elucidate determinants of thermostability and folding pathways of hyperthermophilic proteins using the â-glucosidase from *Pyrococcus furiosus* as a model system. Using fluorescence and circular dichroism (CD) spectroscopy, we have characterized the thermostability and folding pathway of pyrococcal â-glucosidase at 90°C. The chemical denaturation profiles from both spectroscopic methods reveal that this homotetrameric protein unfolds via a three-state pathway with a stable intermediate species at 90°C and ÄG° = 16 kcal/mol. From this data, *P. furiosus* â-glucosidase is one of the most stable proteins yet characterized. Based on the concentration dependence of the stability of the native and intermediate forms and high temperature native gel electrophoresis, we have developed a model for the denaturation of â-glucosidase in which the tetramer dissociates to folded dimers, followed by the coupled dissociation and denaturation of the dimers to unfolded monomers. The extremely high stability is derived from a combination of both oligomer interactions and subunit folding. This stability analysis ultimately provides a platform to compare the stability of homologues from different environments.

S6B:1

EXTREME STABILITY OF A SULFOLOBUS GENOME: EXPERIMENTAL MEASUREMENT AND MECHANISTIC IMPLICATIONS

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The extremely high temperatures required by hyperthermophilic archaea for optimal growth greatly accelerate the spontaneous decomposition of DNA and raise questions about the ability of these archaea to replicate their genomes over many generations without error. Fluctuation analysis using a well-defined, sensitive genetic selection has measured the rate of spontaneous mutation for a 1400-bp region of the Sulfolobus acidocaldarius genome, and sequence analysis of more than 100 independent mutants has revealed the spectrum of changes that occur. The results indicate that this thermo-acidophile exhibits one of the lowest genomic mutation rates yet observed for any DNA genome. When the frequencies of different mutational events in S. acidocaldarius are compared to those observed in other organisms and analyzed with respect to sequence context, additional distinctive features of genetic fidelity in S. acidocaldarius emerge. The resulting pattern suggests a comparatively slow, incremental process of genome evolution in which slipped-strand events during DNA synthesis generate much of the initial variation upon which natural selection subsequently acts.

S6B:2

GLOBAL REGULATION BY HYDROGEN LIMITATION IN METHANOCOCCUS MARIPALUDIS

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Hydrogenotrophic methanogens use hydrogen to reduce carbon dioxide to methane. We have used the model species *Methanococcus maripaludis* to study the global regulatory response to hydrogen limitation. Continuous culture allowed us to control growth rate and cell density under defined nutrient conditions. We are using expression arrays to compare the effects of hydrogen limitation to the effects of leucine limitation (using a leucine auxotrophic mutant) and phosphate limitation. Initial results indicate that some of the hydrogenase genes are expressed at increased levels under hydrogen-limitation, as well as genes for formate dehydrogenase, flagellum synthesis, and certain steps in methanogenesis. In another experiment we have compared gene expression in wild type *M. maripaludis* with a mutant in an energy-coupling, membrane-bound hydrogenase, Ehb. Two enzymes playing central roles in anabolic carbon assimilation were present at lower levels in the mutant compared to the wild type, as supported by array analysis and differential protein levels for multiple subunits. These enzymes were carbon monoxide dehydrogenase/acetylCoA synthase, and pyruvate oxidoreductase, which catalyze carbon dioxide fixation to acetylCoA and pyruvate, respectively. Each of these anabolic steps is believed to require low potential electrons derived from H_2 via the Ehb system. In addition, an AMP-forming acetylCoA synthetase, which catalyzes the first step in acetate assimilation, was expressed at markedly lower levels in the mutant. Our measurements of differential protein levels are part of an ongoing effort to develop a system for high-coverage quantitative analysis of the *M. maripaludis* proteome. Isotope-labeled samples were prepared by growth of cultures with either $^{14}NH_4^+$ or $^{15}NH_4^+$. Each sample was extracted, proteolytically digested, and run twice on a multidimensional LC-MS-MS system. Peptide identities were determined by computational comparison of collision spectra with the annotated genome sequence, and relative peptide abundances were calculated from the intensities of molecular ion spectra. Protein ratios were calculated based on peptide-to-peptide ^{14}N:^{15}N ratios. Our results indicate that the approach can be used for the global measurement of relative protein levels in small-genome organisms.

S6B:3

MOLECULAR ANALYSIS OF A HALOFERAX VOLCANII HMG COA REDUCTASE MUTANT STRAIN

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The moderate archaeal halophile, *Haloferax volcanii*, is currently being used as a model system to examine salinity-mediated gene regulation. To this end, a number of salt-sensitive mutant strains of *H. volcanii* ill-equipped to grow at NaCl conditions greater than 2M have been created via chemical mutagenesis to begin examining both the molecular and genetic mechanisms used by this extremophile to combat the dessicating effects of hypersalinity. One strain we are currently examining, HvM7, has a mutation that leads to a number of interesting phenotypic changes that include elongated cell morphology and altered membrane integrity (as witnessed by TEM analysis). One-dimensional SDS-PAGE analysis of crude extracts prepared from wild-type and mutant *H. volcanii* strains revealed several distinct proteins whose expression differed between the two strains. These proteins were gel-purified and subjected to N-terminal protein sequencing analysis for putative identification of their function. One protein that is conspicuously absent in HvM7 crude extracts has been identified as HMG-CoA reductase, an essential enzyme involved in the synthesis of mevalonate, an integral component of the isoprenoid side chains in Archaeal membranes. Northern analysis was performed to verify transcription levels of this gene from both mutant and wild-type *H. volcanii* and confirmed our findings. Complementation analysis with the wild-type gene restored the mutant strain to a normal phenotype. Finally, protein sequencing analysis has also revealed that HvM7 up-regulates the synthesis of at least three other proteins including RadA, a homolog of bacterial RecA and an integral repair protein in found in Archaea. We are currently investigating the mechanisms underlying this regulation.

S6B:4

GENOME OF THE HALOPHILIC BACTERIUM *SALINIBACTER RUBER.*

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Until recently, hypersaline environments were considered to be the exclusive preserve of halophilic archaea, specialists at coping with these extreme conditions. Thus it was most surprising when Rodriguez-Valera and colleagues discovered that many of the prokaryotic inhabitants of crystallizer ponds correspond to a true bacterium of the *Cytophaga* cluster. Representative strains of this cluster have now been isolated from several locales, and are described as the new species *Salinibacter ruber. S. ruber* is an aerobic heterotroph bacterium that grows readily in pure culture and produces red-pigmented colonies on plates. The bacterium exhibits many "haloarchaeal" physiological properties that may be the result

of a remarkable convergence, an extensive inter-domain gene transfer or (most likely) both. The goal of this project is to sequence, annotate and analyze the entire 3.7 Mb genome of *S. ruber*. Two libraries (one small and one medium insert library) were sequenced to 8.7X coverage by the random shotgun method. A total of 40,904 sequences (overall success rate of 94.1%) were obtained and assembled, using Celera Assembler, into 40 contigs (size ranging from 1.14 Mbs to less than 5 Kbs). TIGR Bambus was then used for scaffolding based on mate-pair information, leading to 24 scaffolds, with 15 sequencing gaps (linking information between contigs available) and 10 physical gaps (no linking information available) remaining. Significant aspects of the *S. ruber* sequencing, gene annotation and genome analysis, as well as comparison with other halophilic archeal species will be presented.

S6B:5

FUNCTIONAL GENOMICS OF LOW TEMPERATURE/HIGH PRESSURE ADAPTATION

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The complete genome sequence of the psychrotolerant, moderately piezophilic, deep-sea bacterium Photobacterium profundum strain SS9 has recently been completed. In this presentation comparative and functional genomic experiments will be described which are helping to define the genes involved in adaptation to low temperature and/or high pressure. These experiments include comparative genomics within P. profundum species which differ in temperature/pressure adaptation, transcriptome studies at 0.1, 28 and 45 MPa, 4° C and 15° C, and global surveys of transposon mutants defective in growth at low temperature or high pressure. Additional experiments planned in the short term include an exploration of the SS9 proteome and the construction of specific mutants by targeted gene disruption. At this juncture it appears that high pressure adaptation requires modulation of membrane and cell wall biosynthesis, amino acid utilization, pH homeostasis, the presence of multiple cytochrome and F1F0 ATPase systems as well as particular sets of isozymes. Low temperature adaptation requires changes in membrane structure, nucleic acid processing, protein secretion, glycolysis and TCA cycle enzyme expression, protein turnover and translation. The significance of the above experiments to uncovering basic themes in cold deep-sea adaptation will be discussed.

S6B:6

EXPLORING THE *SULFOLOBUS SOLFATARICUS* PROTEOME BY TWO DIMENSIONAL ELECTROPHORESIS AND LC-LC-MS-MS

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The hyperthermophilic crenarchaeon *Sulfolobus solfataricus* has been an attractive model organism since its isolation in the early nineteen eighties, and the completion of its genome sequence in 2001 has only increased its popularity. However, few functional genomic studies have been conducted to exploit the information embedded in the genome. Here, we present a proteomic study of global expression profiles of *S. solfataricus* grown on glucose, tryptone and arabinose as the carbon source. We have used both gel-based (two-dimensional electrophoresis) and liquid chromatography based techniques (cation exchange in combination with reversed phase) for protein and peptide separation in order to increase the proteome coverage through identification by electrospray ionisation tandem mass spectrometry (LC-ESI-MS-MS). We have identified peptides corresponding to > 500 ORFs using these techniques. We have successfully introduced stable isotopes into proteins in order to quantitate protein expression. This was done by growing the organism on ^{15}N enriched ammonium sulfate (metabolic labeling), and by derivation to labeled linkers *in vivo* (e.g. ICAT). Peptides become visible as two distinct peaks on the mass spectrometer. The peptides were quantified by calculating the ratio between the intensity of the "heavy" and the "light" peak. Using these techniques we found evidence for a number of up and downregulated proteins. With this proteomic information, we have been able to demonstrate the functional reconstruction of a number of metabolic pathways, such as Entner-Doudoroff Pathway and the Tricarboxylic Acid Cycle

1

BIOCHEMICAL CHARACTERIZATION OF THE METHANOTHERMOBACTER THERMAUTOTROPHICUS MCM HELICASE N-TERMINAL DOMAINS

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Minichromosome maintenance (MCM) helicases are ring-shaped complexes that play an essential role in archaeal and eukaryal DNA replication by separating the two strands of chromosomal DNA to provide the single-stranded substrate for the replicative polymerases. The N-terminal portion of the MCM helicase from the archaeaon Methanothermobacter thermautotrophicus was shown to be involved in multimer formation, single-stranded DNA (ssDNA) binding, and may play a role in regulating helicase activity. The three-dimensional structure of the protein revealed a three domain structure (A, B and C). A detailed characterization of the N-terminal region was performed using biochemical and biophysical approaches. It was demonstrated that domain C, located adjacent to the helicase catalytic domains, is required for protein multimerization. Domain B is the main contact region with ssDNA, probably via a zinc-finger motif located in the domain. Domain A may play a regulatory role. Furthermore, while oligomerization is not essential for ssDNA binding and ATPase activity, the presence of domain C is essential for helicase activity. These observations provide insight for the structure and function relationships of the MCM complexes from other archaeal and eukaryal organisms.

2

DISCOVERY AND CHARACTERIZATION OF THE FIRST THERMOSTABLE 5' POLYNUCLEOTIDE KINASE - 3' PHOSPHATASE FROM THE RM378 BACTERIOPHAGE

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A novel polynucleotide kinase (PNK) gene from the thermophilic bacteriophage RM378 that infects the thermophilic eubacterium *Rhodothermus marinus* was identified, expressed and characterised. The RM378 PNK showed very limited sequence similarity to the 5' kinase domain of the T4 phage PNK, and apparent homology was not evident within the 3' phosphohydrolase domain. In addition, the domain order of the RM378 PNK was opposite to that of T4 PNK. The RM378 phosphohydrolase domain showed some similarity to the bacterial poly(A) polymerase family, with characteristic sequence motifs of HD domains in the diverse superfamily of metal dependent HD phosphohydrolases. The RM378 PNK enzyme showed 5' kinase activity on RNA, single- and double-stranded DNA from 50-80°C. It also showed phosphohydrolase activity on cAMP and less activity on 3'TMP. These findings imply functional analogy to T4 PNK and along with recently described RNA ligase from the RM378 bacteriophage, suggest that the bacteriophage RM378 has to counter similar defense mechanism in *R. marinus* as T4 phage in *Escherichia coli*. This is the first thermostable polynucleotide kinase reported and described from a thermophilic bacteriophage.

3

ISOLATION AND CHARACTERIZATION OF A THERMOSTABLE RNA LIGASE 1 WITH HIGH SSDNA LIGASE ACTIVITY FROM *THERMUS SCOTODUCTUS* BACTERIOPHAGE TS2126

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We have recently identified a novel thermophilic bacteriophage designated TS2126 that infects the thermophilic eubacterium *Thermus scotoductus*. The bacteriophage was isolated and its genome sequenced and analyzed. One of the annotated open reading frames showed homology to T4 RNA ligase 1, an enzyme of great importance in molecular biology due to its ability to ligate single-stranded nucleic acids. The gene was cloned, and the expressed recombinant protein purified and characterized. The recombinant enzyme was shown to ligate single stranded nucleic acids in an ATP-dependent manner and was moderatly thermostable. It presented extremely high activity and high ligation efficiency. It is usable for various molecular biology applications including RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). The TS2126 RNA ligase catalysed both inter and intra-molecular ssDNA ligation to over 50% completion in a few hours at 50-75°C, but had preference for intra-molecular ligation of both RNA and ssDNA. The properties of this new RNA ligase makes it very interesting for processes like adaptor ligation for PCR methodologies and single stranded solid phase gene synthesis.

4

SCREENING AND ISOLATION OF NOVEL THERMOSTABLE DNA POLYMERASES FROM COMPLEX BIOMASS USING GENEMINING™

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In order to explore the diversity of bacterial DNA polymerases from hot spring biomass, we extracted the total DNA from a complex hot spring biomass samples. We used the GENEMINING™ gene retrieval methodology to clone, isolate and screen number of thermostable DNA polymerases for desired properties. This was done by PCR with degenerate primers from the conserved polymerase I (*polA*) active sites and arbitrary primers, allowing us to clone and fully DNA sequence the DNA polymerase

genes without construction of DNA libraries. DNA polymerase genes were selected for further studies, based on the sequence phylogeny of newly retreived and known DNA polymerase sequences. Total of ten DNA polymerases were selected and expressed in in *E. coli*. After purification the DNA polymerases were characterized using standard techniques and desired properties like thermostability, incorporation of modified nucleotides, and processivity. Experimental data and novel properties of some of the new thermostable DNA polymerases will be presented.

5

COMPARATIVE KINETICS OF NUCLEOTIDE ANALOG INCORPORATION BY VENT DNA POLYMERASE

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Comparative kinetic and structural analysis of a variety of polymerases has revealed both common and divergent elements of nucleotide discrimination. Although the parameters for dNTP incorporation by the hyperthermophilic archaeon Family B Vent DNA polymerase are similar to those previously derived for Family A and B DNA polymerases, parameters for analog incorporation reveal alternate strategies for discrimination by this enzyme. Discrimination against ribonucleotides is characterized by a decrease in the affinity of NTP binding and a lower rate of phosphoryl transfer, whereas discrimination against ddNTPs is almost exclusively due to a slower rate of phosphodiester bond formation. Unlike Family A DNA polymerases, incorporation of acyNTPs by Vent DNA polymerase is enhanced over ddNTPs via a 50-fold increase in phosphoryl transfer rate. Furthermore, a mutant with increased propensity for nucleotide analog incorporation (VentA488L DNA polymerase) has unaltered dNTP incorporation, while displaying enhanced nucleotide analog binding affinity and rates of phosphoryl transfer. Based on kinetic data and available structural information from other DNA polymerases, we propose active site models for dNTP, ddNTP and acyNTP selection by hyperthermophilic archaeal DNA polymerases to rationalize structural and functional differences between polymerases.

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CLONING, SEQUENCING, EXPRESSION AND BIOCHEMICAL CHARACTERIZATION OF THE FAMILY B DNA POLYMERASE AND PCNA FROM A RAINBOW HYDROTHERMAL VENT FIELD ARCHAEON

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A hyperthermophilic strain, designated OGL-20, was isolated from mud and rock samples collected at the Rainbow hydrothermal vent field (North Atlantic Ridge) at a depth of 2300 m. Strain OGL-20 was identified as an archaeon belonging to the *Thermococcus* genus according to phylogenetic analysis based on the 16S RNA gene sequence. Parts of the family B DNA polymerase and PCNA (polymerase sliding clamp) genes were amplified using primers based on conserved sequences among related species. Sequences of gene termini were then determined using modified versions of the vectorette-PCR and RSO-PCR techniques respectively, allowing amplification of the full-length coding regions. The DNA polymerase gene contained an intein-coding sequence which was subsequently removed by the overlap extension method. Both genes were cloned in pCR-T7-TOPO expression vectors and expressed in *Escherichia coli* BL21-Rosetta. The mature forms of the DNA polymerase and PCNA have been purified to homogeneity as determined by SDS-PAGE analysis through heat treatment followed by three chromatographic steps. The aminoacid sequence of the DNA polymerase showed highest homology to the DNA polymerase sequence from *Thermococcus sp.* 9°N-7 with 90.4% identity, while for the PCNA, the closest homologue among available sequences is from *Thermococcus fumicolans* with 79.5% identity. Biochemical and enzymatic properties were compared to those of other thermostable enzymes. The DNA polymerase was found to be remarkably suited for PCR applications. The influence of PCNA on polymerase processivity was also investigated.

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THE THREE CDC6-LIKE FACTORS OF *SULFOLOBUS SOLFATARICUS* PROMOTE BINDING OF MCM TO DNA

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Comparative genomics indicates that the archaeal replication machinery is a simplified version of the eukayotic one, thus suggesting that the replication mechanisms in Archaea could be similar. Archaea have genes coding for putative homologous of several eukaryotic replication proteins in their genomes, including the initiation factors Cdc6 and MCM. We investigated the biochemical properties of the three Cdc6 factors (referred to as SsoCdc6-1, -2 and -3) from *Sulfolobus solfataricus*. We first demonstrated that the three SsoCdc6 factors bind DNA with a preference for DNA molecules that mimic early replication intermediates. Moreover we observed that SsoCdc6-3 binds DNA with a higher affinity in comparison with the other two SsoCdc6 factors. As well as *Methanothermobacter thermautotrophicus* Cdc6 proteins, SsoCdc6-1 and -2 (1) are able to autophosphorylate *in vitro* while SsoCdc6-3 does not possess this activity. We also demonstrate that each SsoCdc6 factor stimulates binding of the homo-hexameric SsoMCM helicase (2) to bubble-containing DNA molecules. Using ÄC, a truncated form of SsoCdc6-2 (residues 1-297), which lacks DNA binding activity (3), we could demonstrate that the stimulatory effect of SsoCdc6-2 does not require its DNA binding function. ATP is also not needed for this activity since a SsoCdc6-2 Walker A mutant, which does not bind ATP, is still able to stimulate SsoMCM binding to DNA. Immuno-precipitation experiments indicate that SsoCdc6-2 and SsoMCM physically interact and this interaction does not involve the last 102 amino acids of SsoCdc6-2 polypeptide chain since ÄC still retains this capability. We also show that this interaction does not require the first 268 amino acids of SsoMCM polypeptide chain since SsoMCM-Ä268 inhibits SsoCdc6-2 binding

to DNA and physically interacts with it. These findings provide the first *in vitro* biochemical evidence of a functional interaction between MCM and Cdc6 factors and have important implications for the understanding of the Cdc6 biological function and of the evolution of the DNA replication initiation process in the more complex eukaryotic organisms.

1) De Felice *et al.*, 2003, *J. Biol. Chem.* **278**, 46424-31.
2) Carpentieri *et al.*, 2002, *J. Biol. Chem.* **277**, 12118-27.
3) De Felice *et al.*, 2004, *Biochem. J.*, in press.

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DIFFERENCES IN THE SALT DEPENDENCE OF DNA BINDING BY *THERMUS AQUATICUS* AND *ESCHERICHIA COLI* DNA POLYMERASES

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DNA binding properties of the Pol 1 type DNA polymerases from *Thermus aquaticus* (Taq, Klentaq) and *Escherichia coli* (Klenow) have been examined as a function of KCl , $MgCl_2$, K-acetate, and K-glutamate. Full length Taq and its Klentaq "large fragment domain" behave similarly in all assays. The two different species of polymerases bind DNA with sub-micromolar affinities in very different salt concentration ranges. Consequently, at similar salt concentrations the binding of Klenow is ~ 3 kcal/mol (150X) tighter than that of Taq/Klentaq to the same DNA. Linkage analysis in KCl and KAc reveals a net release of 2-3 ions upon DNA binding of Taq/Klentaq, and 4-5 ions upon binding of Klenow. K-glutamate significantly reduces the linked ion release upon binding for Klenow but not for Klentaq. *E. coli* and *T. aquaticus* are believed to predominantly contain different glutamate isomers, but use of D- versus L-isomers of glutamate does not significantly alter any of the glutamate effects observed for either polymerase. The identity of the anion alters the relative affinity of DNA binding similarly for both species of polymerase, with binding being tightest in glutamate and weakest in chloride. Linkage analysis of binding as a function of the divalent cation magnesium ($MgCl_2$) reports the ultimate release of ~1 Mg^{+2} ion upon complex formation. However, the $MgCl_2$ dependence for Klenow, but not Klentaq, shows two distinct phases. In 10mM EDTA, both polymerase species still bind DNA, but their binding affinity is significantly diminished, Klenow more than Klentaq. In summary, the two polymerase species, when binding identical DNA, differ substantially in their sensitivity to the salt concentration range, bind with very different affinities when compared under similar conditions, release different numbers of ions upon binding, respond significantly differently to glutamate anions, and differ in their interactions with divalent cations. These differences in salt sensitivity and specificity indicate that the ionic regulation of the two species of polymerases are different *in vivo*.

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AMINO ACID RESIDUES OF MCM DNA HELICASE OF SULFOLOBUS SOLFATARICUS INVOLVED IN DNA BINDING

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The MCM-like protein of the crenarchaeon Sulfolobus solfataricus (SsoMCM) was produced in recombinant form and extensively characterized in our laboratory. SsoMCM forms homo-hexamers in solution (470 kDa), possesses ATPase and DNA helicase activity, physically and functionally interacts with SsoSSB and SsoCdc6 factors (1, 2). More recently we have found by atomic absorption measurements that the protein binds Zn2+ ions, as also determined for the Methanobacterium thermoautotrophicum MCM (MthMCM). In addition we have demonstrated by electrophoretic mobility-shift assays (EMSAs) that SsoMCM binds a variety of synthetic oligonucleotides: molecules containing either bubble or fork structures, blunt duplexes and single stranded DNA. Interestingly, SsoMCM shows a clear preference for DNA molecules that mimic early replication intermediates. In order to investigate the protein structure/function relationships, a truncated form of SsoMCM has been produced and characterized that lacks the first 268 amino acid residues (SsoMCM D268). This region includes the Cys2 Zn-binding motif and a cluster of basic residues that are thought to be involved in DNA-binding based on the x-ray structure of the MthMCM N-terminal fragment (3). We found that SsoMCM D268 forms homo-hexamers in solution, retains ATPase activity, but is completely unable to bind DNA, as assayed by EMSAs. In order to more precisely define the region implicated in DNA-binding we have performed a site-directed mutagenesis analysis of SsoMCM. The amino acid residues to be substituted were selected on the basis of a pairwise alignment between the Sso and MthMCM protein sequences and by inspecting the MthMCM N-terminal fragment three-dimensional structure. SsoMCM single-residue mutants were produced where K129, K134, H146, K194 were substituted with alanine. These positively charged residues (except for H146) are conserved between Sso and MthMCM and in the x-ray structure of the MthMCM they are located on the internal surface of the channel proposed to encircle DNA (3). The biochemical characterization of these SsoMCM mutants is being carried out in our laboratory. Results of this analysis will be presented.

1) Carpentieri F. et al. 2002 J Biol Chem. 277: 12118-27.
2) De Felice M. et al. 2003 J Biol Chem. 278: 46424-31.
3) Fletcher R. J.et al. 2003 Nat Struct Biol. 10: 160-7.

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A NOVEL HELICASE RELATED TO REPLICATION FORK REPAIR IN ARCHAEA

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Homologous recombination plays important roles for DNA transactions including several DNA repair process and generation of genetic diversity in the living cells. Holliday junction (HJ) is an important intermediate during homologous recombination process. We identified the Holliday junction-resolving enzyme in Archaea and designated HjcÅ@Å@previously (1-5). To identify the branch migration activity in archaea, we fractionated P. furiosus cell extracts by several chromatographies and assayed for ATP-dependent resolution of synthetic Holliday junctions. The target activity was identified in the column fractions and the optimal reaction condition for the activity was determined using the partially purified fraction. Then, we succeeded to clone the corresponding gene by screening of a heat-stable protein library of P. furiosus. The gene, hjm (Holliday junction migration), encodes a protein composed of 720 amino acids. Hjm protein is conserved in Archaea and has some sequence similarity to a human helicase HEL308, which is related to a repair process, whereas in bacteria and yeast, any similar sequence was not found. We made an overproduction system of Hjm in E. coli and prepared a highly purified protein. Biochemical characterization of Hjm helicase showed that Hjm has binding affinity to fork-related Y-structure DNA and unwinds the double-strand regions in addition to the Holliday junction. Furthermore, genetic analyses showed that Hjm partially complemented the RecQ function in E. coli recQ mutant cells. These results suggest that Hjm may be a functional counterpart of RecQ in Archaea and is required for the maintenance of genome integrity. Structural and functional analyses are in progress to understand more detailed function of Hjm in the DNA transactions.

1. K. Komori, S. Sakae, H. Shinagawa, K. Morikawa, and Y. Ishino (1999) Proc. Natl. Acad. Sci. USA 96, 8873-8878.
2. H. Daiyasu, K. Komori, S. Sakae, Y. Ishino, and H. Toh (2000) Nucleic Acids Res. 28, 4540-4543.
3. K. Komori, S. Sakae, R. Fujikane, K. Morikawa, H. Shinagawa, and Y. Ishino (2000) Nucleic Acids Res. 28, 4544-4551.
4. K. Komori, S. Sakae, H. Daiyasu, H. Toh, K. Morikawa, H. Shinagawa, and Y. Ishino (2000) J. Biol. Chem. 275, 40385-40391
5. T. Nishino, K. Komori, D. Tsuchiya, Y. Ishino, and K. Morikawa (2001) Structure 9, 197-204.

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FUNCTIONS OF THREE PCNAS IN THE DNA REPLICATION PROCESS IN AEROPYRUM PERNIX

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PCNA (Proliferating Cell Nuclear Antigen) is a well-known multifunctional protein, involved in eukaryotic DNA transactions. The homotrimeric PCNA ring encircles double-stranded DNA within its central hole and tethers many proteins on DNA. Archaea, the third domain of life, has PCNA homologs, and some of them have been biochemically characterized as the clamp for DNA polymerases to synthesize DNA strand processively. Å@ Three genes encoding PCNA-like proteins have been found in the genome sequences of crenarchaeal organisms, and the share of the roles of them in the cells are now very interesting to be understood. We presented previously that all three PCNAs from Aeropyrum pernix stimulate DNA synthesis activities of the two identified DNA polymerases, Pol I and Pol II, in this organism in vitro (1). Then, Bell and co-workers presented that the three PCNA proteins in S. solfataricus form a heterotrimer and each protein interact with Pol, Lig, and FEN, respectively. They speculated that the heterotrimeric PCNA works for the efficient lagging strand synthesis by the share of interaction with other essential proteins in DNA replication (2). To know more precisely about specificity of each PCNA in terms of interactions with DNA replication proteins, we did the DNA synthesis assays using various conditions. From these results, as well as the structural prediction, we will discuss the roles of each PCNA in the DNA transactions in Crenarchaeota.

1. Daimon, K., Kawarabayasi, Y., Kikuchi, H., Sako, Y., and Ishino, Y. (2002) J. Bacteriol. 184, 687-694.
2. Dionne, I., Nookala, R.K., Jackson, S.P., Doherty, A.J., Bell, S.D. (2003) Mol. Cell 11275-11282.

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SS-LRPB, A NEW TRANSCRIPTION REGULATOR FROM *SULFOLOBUS SOLFATARICUS*, FORMS HIGHER ORDER NUCLEOPROTEIN COMPLEXES WITH THE OWN CONTROL REGION

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The archaeal basal transcription apparatus is a simplified version of its eukaryotic counterpart. Intriguingly, it seems to be regulated mostly by Bacteria-like transcription regulators, an observation which reveals a hybrid situation (1). Most of the to date characterized archaeal transcription regulators belong to the Leucine responsive Regulatory Protein (Lrp)-family, a family with members in both Archaea and Bacteria (2). A new member of this family is Ss-LrpB from *Sulfolobus solfataricus*. We present an elaborate *in vitro*

characterization of the interaction of Ss-LrpB with its own control region, which is suggestive of autoregulation, and demonstrate that the operator shows an intrinsic bend and is further deformed upon protein binding. Binding occurs cooperatively at three regularly spaced, palindromic binding sites with a similar sequence (Box1, Box2 and Box3), as shown by various mobility shift-, footprinting-, and premodification binding interference experiments. 'In gel' Cu-phenantroline footprinting suggested that the outer Box1 and Box3 both need to be bound by Ss-LrpB before the middle Box is bound as well. Ss-LrpB interacts with two major groove segments and the intervening minor groove of each binding site; all contacts are aligned on one face of the helix. Presumably, both protein-protein interactions between adjacent bound protein oligomers as well as major DNA deformations upon protein binding play an important role in complex formation. A molecular model of this higher order Ss-LrpB/DNA complex will be proposed. The most downstream Box1 shows an overlap of 1 bp with the factor B recognition promoter element (BRE). Nevertheless, simultanous binding of Ss-LrpB and the *Sulfolobus* transcription factors TBP and TFB to the promoter/operator region is possible, as shown by mobility shift- and in gel footprinting-assays. Therefore, we hypothesize that Ss-LrpB might positively regulate its own expression, at least under certain conditions. Possibly, there exists a 'switch' between positive and negative autoregulation depending on Ss-LrpB concentration.

(1) Bell, S.D. & Jackson, S.P. (2001). *Curr Opin Microbiol* **4**, 208-213.
(2) Brinkman, A.B., Ettema, T.J.G., de Vos, W.M. & van der Oost, J. (2003). *Mol Microbiol* **48**, 287-294.

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EXPRESSION PROFILES OF THE HYPERTHERMOPHILIC BAROPHILE *METHANOCOCCUS JANNASCHII* IN THE P-T PLANE: A MEANS FOR DISCOVERY OF NOVEL GENE PRODUCTS

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Temperature shock of the hyperthermophilic methanarchaeon *Methanococcus jannaschii* from its optimal growth temperature of 85°C to 95°C resulted in up-regulation of genes encoding chaperones including the á subunit of a putative prefoldin, which may comprise a novel element in the protein processing pathway in *M. jannaschii*. Very different responses were observed upon cold shock to 65°C. These included up-regulation of a gene encoding proteins involved in transcription and translation including an RNA helicase, and up-regulation of genes coding for transport proteins. Based on the overall responses to the two temperature shocks, pathways for processing nascent or unfolded proteins in *M. jannaschii* have been proposed and novel products have been characterized. Responses to pressure have also been explored, by determining expression profiles for growth at pressures of 7 atm and 500 atm. These data have revealed possible adaptation mechanisms underlying barophilic growth at high temperatures.

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TRANSCRIPTION ANALYSIS OF THE ROD-SHAPED VIRUSES SIRV1 AND SIRV2 OF THE HYPERTHERMOPHILIC ARCHAEON *SULFOLOBUS*

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The double-stranded DNA genomes of the crenarchaeal rudiviruses SIRV1 (32 kb) and SIRV2 (35 kb), that use *Sulfolobus* species as host, have previously been sequenced completely. We here present the mapping of transcriptional start sites, as well as the analysis of gene expression at different time points after infection of *Sulfolobus islandicus* host cells. Transcription of both genomes starts simultaneously at multiple sites spread over the total genomes, from both strands. The earliest time point when viral transcripts could be detected in cells was 30 min after infection. At this time point all the viral genes, except one, were transcribed. Many genes are clustered and appear to be transcribed as polycistronic messengers. Although the coat protein-encoding gene is initially also transcribed as a polycistronic messenger, an abundant monocystronic transcript of this gene was detected 2-3 hrs after infection, just before assembly of viral particles. The expression of a single gene, located adjacent to the coat protein gene, is upregulated at the late phase of infection, suggesting it might be involved in the specific processing and activation of the coat protein messenger. Start sites of thirteen transcripts from the SIRV1 genome have been mapped by primer extension, and promoter sequences were identified. Similar to host promoters, these viral promoters all contain potential binding sites for the archaeal transcription factors TATA Binding Protein (TBP) and Transcription Factor B (TFB). Interestingly, however, the majority of the promoters of SIRV1 (70%) and SIRV2 (65%) appear to have distinct promoter structures, suggesting the involvement of alternative transcription factors.

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DEVELOPMENT OF GREEN FLUORESCENT PROTEIN-BASED EXPRESSION VECTORS TO IDENTIFY REGULATED PROMOTERS OF *HALOFERAX VOLCANII*

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Over the past several years, a considerable number of regulated components have been discovered among the phylogenetically distinct group of haloarchaea. These components are influenced by such factors as oxygen tension, light intensity, osmolarity, acetate concentrations, nitrate levels, and availability of growth substrates including glucose and fructose. Non-constitutive metabolic enzymes account for the majority of these components and include enzymes involved in acetate activation (AMP-producing acetyl-CoA synthetase in *Haloferax volcanii*) as well as hexose metabolism via modified Embden-Meyerhof and Entner-Doudoroff pathways for

fructose and glucose, respectively. Another major group of regulated components is represented by cellular transport systems such as a sodium gradient-dependent fructose uptake system and ABC transporter for nitrate respiration, both in *H. volcanii*. To date, the induction of these various components has not been investigated in significant detail at the genetic level with the exception of the *bop* (Bacterioopsin) gene cluster and, specifically, the trans-acting factor produced by the *bat* gene in *Halobacterium sp.*, induced by low oxygen tension and elevated light intensity. In this study, we generated a series of green fluorescent protein-based vectors (suicide and replicating plasmids) for use in the identification of regulated promoters of *H. volcanii*. Current efforts are focused on screening *H. volcanii* genomic DNA fragments cloned upstream of the promoterless green fluorescent protein reporter gene of these vectors. 2D-PAGE proteome maps of cells grown under different conditions (*e.g.* glucose- vs. fructose-supplemented media) are also being developed to identify variably expressed proteins, which may be controlled at the transcriptional level. Identification of such proteins may allow us to make use of upstream promoter sequences to control gene expression through recombinant biotechnology.

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GLOBAL TRANSCRIPTOME RESPONSE OF *DEINOCOCCUS RADIODURANS* TO CHRONIC IONIZING RADIATION AND PEROXIDE STRESS

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The bacterium *Deinococcus radiodurans* is extremely resistant to ionizing radiation. How this organism is able to grow under chronic gamma-radiation or recover from acute doses greater than 10,000 Gy is unknown. Previously reported transcriptome dynamics of *D. radiodurans* recovering from a very high dose of acute radiation (15 kGy) using a whole genome microarray covering ~94% of its predicted genes supports the view that *D. radiodurans*' extreme radiation resistance phenotype is complex, likely determined by an assortment of protection and DNA repair systems. In this study we used whole genome transcriptome profiling to examine *D. radiodurans* grown in the presence of chronic irradiation (50 Gy/hour) in rich medium, or exposed to hydrogen peroxide (H_2O_2). Unexpectedly, no marked differences in expression were observed for cells growing under chronic radiation compared to non-irradiated control cells. This supports that oxidative stress levels are not significantly different in *D. radiodurans* under chronic radiation than in non-irradiated cells. Therefore, *D. radiodurans* may contain protection systems not present in radiation sensitive bacteria such as *Shewanella oneidensis*, which are extremely radiation sensitive and unable to grow under chronic radiation. As expected, *D. radiodurans* is very resistant to exposure to H_2O_2 and expression profiles of cells recovering from 3 different concentrations of H_2O_2 will be presented.

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FATE OF THE *METHANOBACTER THERMAUTOTROPHICUS* ARCHAEAL GENERAL TRANSCRIPTION FACTORS TBP AND TFB FOLLOWING TRANSCRIPTION INITIATION

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Archaeal RNA polymerases (RNAPs) and basal transcription factors TATA-box binding protein and TFB are closely related to their eukaryotic counterparts, whereas most of the known and predicted archaeal transcription regulators resemble bacterial regulators. Initiation of archaeal transcription requires the promoter-directed assembly of a pre-initiation complex containing minimally TBP, TFB and RNAP. In vitro, this process has been shown to be either enhanced by activators or blocked by repressors that bind upstream regulatory sequences. We have used two approaches to determine the fate of the TBP and TFB components of the pre-initiation complex following transcription initiation in vitro using a system derived from the thermophilic archaeon, *Methanothermobacter thermautotrophicus*. Templates of different length were constructed that contain the TATA-box region from the *hmtB* archaeal histone-encoding gene. One template was incubated with TBP, TFB and RNA polymerase and then, after removal from the reaction mixture and washing, the resulting complexes were mixed with a second template in a reaction mixture that lacked either TBP or TFB. Transcription was then allowed to initiate at 58°C by addition of NTPs, and the transcripts synthesized were determined. Transcripts were synthesized from the second template in reaction mixtures lacking added TFB but not in the absence of added TBP consistent with the release of TFB but not TBP from the first template following transcription initiation. This result was confirmed using antibodies to determine the fate of TBP and TFB following transcription initiation. TBP remained bound to the template DNA but TFB was released from the template DNA after the *M. thermoautotrophicum* RNAP had initiated and transcribed >5 nucleotides. The observation that archaeal TBP remains bound to the promoter following transcription initiation is consistent with homology to the eukaryotic RNAPII system but poses a conceptual problem for transcription regulation based on a competition between repressors and TBP for promoter binding. Furthermore, MOT1, an essential protein in yeast, catalyzes the ATP-dependent dissociation of yeast TBP from RNAPII promoter DNA, but there is no genome sequence evidence that *M. thermautotrophicus*, or in any other Archaeon, contains an archaeal homolog of this eukaryotic basal transcription regulator.

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BLACK YEAST HORTAEA WERNECKII - A NEW EUKARYOTIC HALOPHILIC MODEL ORGANISM

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Hypersaline environments are inhabited by bacteria, few algal species and a new group of eukaryotic extremophiles represented by melanized fungi, so called black yests. The dominant species among them is the extremely halophilic *Hortaea werneckii*, with the capability of growing in media with no salt and up to 5.2 M NaCl concentration. It represents an appropriate model organism to study molecular mechanisms of salt tolerance in eukaryotes. Cellular responses to high salinity were investigated in *H.werneckii* at the level of expression of salt responsive genes and fuctions of their their products. By using a restriction fragment-differential display method we identified eight differentially expressed genes in response to different salt concentration. Functions of their putative products within cellular machinery was proposed. An increase in intracellular glycerol concentration was observed at increased environmental salinities in *H. werneckii*. Expression profile of a gene, coding for a putative glycerol-3-phosphate dehydrogenase (Hwgpd), a key enzyme in glycerol biosynthesis, revealed several-fold increased expression at high salt concentration. This increased expression of Hwgpd gene correlated with the increased activity of HwHog1, a key kinase of HOG signalling pathway, which senses and responds to hyperosmotic stress, and regulates the expression of gpd genes. Since glycerol is abundant at higher salinities, we speculated, that relatively less metabolic flux remains for the late stages of glycolysis and for the Krebs cycle. This assumption was supported with our finding, that production of CO_2 was diminished at higher salt concentration. Increased expression of SOL5, a putative aconitase gene at increased salinity could also be related to this phenomenon. No major changes in intracellular ion concentration occur in *H. werneckii* with the increased external salinity. Although Na^+ to K^+ ratio increases with increased NaCl concentration in the medium, it does not follow the external Na^+ concentration. These data suggest, that halophilic *H. werneckii* is well adapted to changes in external ion concentrations, most probably due to its ability to effectively extrude Na^+ ions. Expression profile of two genes, that could be connected to Na^+ transport, SOL4 and homologue of ENA1, support this hypothesis. Co-ordinated expression of salt-stress responsive genes, correlated to their putative functions, have so far revealed some mechanisms that enable *H. werneckii* to thrive at extremely high NaCl concentration and to adapt to a wide range of NaCl concentrations in its environment.

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COMPARATIVE TRANSCRIPTIONAL PROFILING OF THE HYPERTHERMOPHILIC BACTERIUM *THERMOTOGA MARITIMA* AND A RESISTANT MUTANT UPON EXPOSURE TO THE ANTIBIOTIC CHLORAMPHENICOL

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Hyperthermophilic microbial communities consist of highly diverse groups of archaea and bacteria, which are invariably classified among the deepest branches of the phylogenetic tree. These diverse communities may be the most primitive continuously inhabited ecosystems on Earth. In these niches, coexistence presumably involves antagonistic interactions, in which numerical supremacy is shaped by selective biologically-mediated forces. One of the most studied of these interactions in less thermophilic niches relates to the production of antibiotics such that a competitive advantage accrues to the organisms that can generate them. While this issue has not been studied to any extent in high temperature microorganisms, it has been shown that strains of *Halobacterium sp.* can produce peptide antimicrobials which are active against hyperthermophilic crenarchaeota of the genus *Sulfolobus*. This raises the possibility that similar phenomena exist in hydrothermal environments. The present study focuses on the response of the hyperthermophilic bacterium *Thermotoga maritima* to a common antimicrobial, chloramphenicol. The minimum inhibitory concentration (MIC) to this antibiotic upon incubation at 80°C for 12 hours in SSM-Cellobiose media was found to be 25 ìg/ml. However, thermal decay of the activity of this antibiotic can be seen with a pre-incubation as short as 24 h. Using consecutive passages to increasing concentrations of this antibiotic, it is possible to isolate mutants with an MIC of at least 1mg/ml. In order to investigate the mechanisms associated with these high levels of resistance, transcriptional response experiments using a whole genome cDNA microarray for *T. maritima* were conducted with both wild-type and mutant strains that were challenged with various levels of chloramphenicol. Transcriptional profiles were obtained from mid-exponential cultures showing differences between the mutant and the wild-type strains. In addition, using a 14-liter high temperature fermenter, dynamic response of these two strains upon exposure to 100 ìg/ml of chloramphenicol was monitored to follow time-dependent patterns of gene expression. In addition to addressing aspects of antibiotic resistance in hyperthermophiles, the elucidation of resistance mechanisms could lead to a source of new selectable markers for the development gene delivery systems in these microorganisms.

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TRANSCRIPTIONAL ANALYSIS OF THE EXTREMELY THERMOACIDOPHILIC ARCHAEON *SULFOLOBUS SOLFATARICUS* DURING PYRITE DISSOLUTION PROCESSES

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Sulfolobus solfataricus is an extremely thermophilic crenarchaeon that can thrive in sulfur-rich, highly acidic environments. Its completed genome sequence was found to encode genes related to metal dissolution processes, to existence at atypically low pHs and high temperatures, and to its capacity to hydrolyze various sugars as carbon and energy sources. Of interest here were the mechanisms by which this microorganism solubilizes iron pyrite. These have been investigated from several perspectives focusing on the contributions of direct and indirect mechanisms by which both attached cells and planktonic cells can contribute to the overall iron/sulfur oxidation process. However, comprehensive genome-wide transcriptional analysis of metabolic events in the pyrite dissolution process has yet to be reported. As such, a full-genome oligonucleotide (60mers) cDNA microarray was designed and constructed to facilitate studies examining transcriptional response to medium composition, pH shift and surface attachment as these relate to pyrite oxidation. A mixed effects statistical model was used to confirm proposed mechanisms for pyrite dissolution and to elucidate metabolic features related to this process. Results from these studies will be reported as will insights that support additional annotation of the *S. solfataricus* genome.

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INFLUENCE OF GROWTH PHASE AND GROWTH RATE ON THE TRANSCRIPTOME OF THE HYPERTHERMOPHILIC ARCHEAON *PYROCOCCUS FURIOSUS* GROWN IN BATCH AND CONTINUOUS CULTURE

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Pyrococcus furiosus, a hyperthermophilic archaeon growing optimally at temperatures of 98-100°C, has been studied extensively with respect to its microbial biochemistry and physiology. The availability of its completed genome sequence has enabled functional genomics studies to be carried out focusing on many issues, including sulfur reduction, heat shock response and carbohydrate utilization. However, these studies typically involve batch culture, and the influence of growth phase is usually not considered in the interpretation of results. In view of this, we have used of whole genome cDNA microarray to investigate transcriptional changes that correspond to progression through exponential phase for *P. furiosus* as well as during the transition from exponential to stationary phase. Using a high temperature fermentation system, *P. furiosus* was grown over a 24-hour period in 10L of a sea salts-based complex medium supplemented with 5 g/L of cellobiose. Samples were drawn from the reactor at regular intervals through exponential, stationary and death phases and evaluated for cell density as well as transcriptional state. A loop experimental design was used in conjunction with mixed effects model analysis to follow dynamic transcriptional changes as a function of cultivation time. In addition, continuous culture experiments were also carried out to determine the effect of growth rate on gene expression patterns and to compare with the transcriptome of *P. furiosus* cells grown in batch mode at comparable growth rates. Results will be presented that illustrate the importance of considering growth phase and growth rates effects on interpreting transcriptional response data as well as issues that arise from cultivation mode.

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COORDINATE EXPRESSION OF A MAR-LIKE OPERON AND AN ALCOHOL DEHYDROGENASE GENE CONTRIBUTES TO DETOXIFICATION BY AROMATIC ALDEHYDES IN SULFOLOBUS SOLFATARICUS

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Investigation of mechanisms underlying transcriptional regulation of Sso2536, encoding for an alcohol dehydrogenase gene (adh) in the crenarchaeon S. solfataricus (1) has shown an active 5' flanking region responsive to physiologically relevant DNA binding proteins. In particular, one DNA binding protein, Bald16 (Sso1352), has been identified whose levels are higher when cells are grown in the presence of the toxic benzaldehyde, substrate of the ADH enzyme (2); it has been proposed that this protein could act as a transcriptional activator triggering adh expression to protect cells from an environmental stress due to phenolic-derived aldehydes (3). Bald16 encodes for a putative transcriptional regulator, which has a bacterial homologue belonging to the Mar (Multiple Antibiotic Resistance) family of regulators involved in the control of gene expression of aromatic compound metabolism and antibiotic resistance (4). To better investigate the molecular mechanisms underlying transcriptional regulation in S. solfataricus, with greater attention with respect to defense response upon chemical stress, we analyzed the expression of the bald16 gene in the presence of aromatic aldehydes. Transcriptional analysis of the bald16 gene allowed the identification of a new mar-like locus in S. solfataricus composed of a putative multidrug transporter and the transcriptional regulator downstream (Sso1351, Sso1352). The genes are transcribed as a polycistronic unit whose expression is sensitive to the addition to the cell growth medium of different aromatic aldehydes. The gene encoding for the transcriptional regulator, has been expressed in E. coli and the recombinant protein purified to homogeneity. The protein is indeed a DNA binding protein, which binds site-specifically to both the adh and bald16 promoters. Western blot analysis revealed an increased Bald16 expression in cell extracts prepared from cells grown in the presence of aromatic aldehydes. These results reasonably strengthen the hypothesis of a resistance mechanism based on the coordinate expression of the

adh gene and the Mar-like operon, in response to stress determined by phenolic-derived materials.
(1) She, Q et al. (2001) Proc. Natl. Acad. Sci. USA. 98, 7835-40.
(2) Cannio, R et al. (1999) FEMS Microbiol. Lett. 170, 31-39.
(3) Fiorentino, G et al. (2003) J. Bacteriol. 185, 3926-3934.
(4) Pedone E et al. (2004) Frontiers in Biosciences, in press.

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COMPARATIVE FUNCTIONAL GENOMICS ANALYSES OF MEDIUM COMPOSITION EFFECTS AND TRANSIENT HEAT SHOCK RESPONSE IN THE MODEL HYPERTHERMOPHILES *THERMOTOGA MARITIMA* AND *PYROCOCCUS FURIOSUS*

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Full genome cDNA microarrays were used to examine steady and transient growth in the model heterotrophic hyperthermophiles *Thermotoga maritima* MSB8 and *Pyrococcus furiosus* DSM 3638. Like *T. maritima*, *P. furiosus* was found to have highly discriminating mechanisms to acquire, process and regulate the utilization of simple and complex sources of carbon, including peptides, chitin, barley glucan, laminarin, cellobiose, maltose and starch. Also, the presence of yeast extract and S^0 in the medium led to numerous transcriptional changes between growth conditions in this organism. In addition to medium composition, the dynamic transcriptional response of *P. furiosus* and *T. maritima* to temperature shifts of 90!105°C and 80!90°C, respectively, was monitored over a 90 minute period following the shift. In addition to the induced expression of genes encoding important molecular chaperones, heat shock response of both organisms up-regulated the expression of genes encoding proteins important to the stabilization and repair of DNA and down-regulated the transcription of genes involved in energy metabolism. However, in contrast to transcriptional analyses of the much-studied mesophiles *Escherichia coli* and *Bacillus subtilis*, neither of these hyperthermophilic species exhibited significant induction of genes related to ATP-dependent proteolysis. Even so, transient response data demonstrated that the mechanisms governing heat shock response in these organisms varied over 90-minutes of thermal stress. Indeed, a subset of genes containing greater than 40% pairwise best similarity had a similar early induction pattern for growth on peptides and maltose in *P. furiosus* and growth on maltose in *T. maritima*. Consequently, both organisms seem to employ similar strategies for dealing with heat shock, even though they originate from separate domains of life.

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HYDROGEN REGULATION OF GLOBAL GENE EXPRESSION IN *METHANOTHERMOBACTER THERMAUTOTROPHICUS*

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The thermophilic archaeon *Methanothermobacter thermautotrophicus* (*M.t.*) synthesizes biomass and generates energy from CO_2 and H_2. In the methanogenesis pathway, CH_4 is synthesized from CO_2 via seven-steps, some of which are catalyzed by different but functionally-equivalent enzymes or isoenzymes depending on the availability of H_2. Previous studies have established that this H_2-dependent regulation occurs at the level of transcription initiation, with individual promoters responding differently under different growth conditions. To determine how H_2-supply determined differences in growth rate effect all gene expression, microarray experiments have been carried out using *M.t.* genome microarrays that have DNA spots that represent >98% of the open reading frames annotated in the *M.t.* genome sequence. The results have confirmed the earlier operon specific northern blot data and have identified many additional transcripts that are present in relatively more or less abundance in cells growing slowly under low, or faster under high H_2-supply conditions. The majority of these transcripts are predicted to encode proteins involved in hydrogen metabolism and methanogenesis, cofactor metabolism, citrate cycle reactions, pyruvate and acetyl Co-A metabolism, electron transport, redox reactions, ion transport and 2-component signal transduction. By correlation of these transcript patterns with the sequences of the intergenic regions upstream of the encoding genes, conserved and consensus sequence motifs have been identified and these are being experimentally investigated as potential binding sites for regulatory proteins. The genome of *M.t.* is bound *in vivo* by archaeal histones into archaeal nucleosomes. The involvement of archaeal nucleosomes in regulating *M.t.* gene expression is also investigated by determining and relating the changes in the locations of archaeal nucleosomes in vivo in *M.t.* cells growing under different conditions with the established changes in global transcript patterns.

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ANALYSIS OF SUBUNITS INTERACTIONS OF THE *PYROCOCCUS FURIOSUS* RNA POLYMERASE

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Archaeal RNA Polymerases (RNAPs) resemble the eukaryotic nuclear RNAPs in complexity and many of their subunits are highly homologous to their eukaryotic counterparts. *Pyrococcus furiosus* RNAP consists of 12 different subunits that range in size from 5.7 kDa to 127 kDa. All 12 subunits were cloned, expressed in *E. coli* and purified. To study interactions of subunits far-western analysis

were performed. Subunit D interacts specifically with the subunits B, L, N and P. A complex of subunits D-N was formed *in vitro*. In addition D and L associated under *in vitro* conditions and copurified with each other during size-exclusion chromatography. Far-western analyses indicated also the expected strong binding of F to E'. These results showed in a fascinating way a great degree of structure similarity when compared with the crystal structure of RNAP II (1) and the reconstituted *Methanococcus jannaschii* RNAP from individual subunits (2). Analyses using mutated or modified subunits will identify the protein sequences and amino acids essential for RNAP structure and functions.

(1) Cramer, P. et al. (2000) Science *288*, 640-649
(2) Werner, F., and Weinzierl, R.O.J. (2002) Mol Cell *10*, 635-646

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COMPARISON OF PYROCOCCUS FURIOSUS TFB1 AND TFB2 FUNCTION IN TRANSCRIPTION INITIATION COMPLEX FORMATION

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The archaeal transcription mechanism can be studied in vitro using promoter DNA, transcription factors TBP and TFB, and RNA polymerase. The P. furiosus genome encodes two TFB-related polypeptides, TFB1 and TFB2. The function of TFB1 in transcription complex formation has been well-characterized, but TFB2 has not been shown to have TFB-related activity. We have purified recombinant TFB2 and examined its activity in promoter-dependent transcription initiation complex formation, using photochemical cross-linking and in vitro transcription assays. We find that TFB2, like TFB1, can recognize promoter-bound TBP and can direct site-specific transcription initiation by RNA polymerase, although the activity of TFB2 in transcription appears significantly lower than TFB1 at most promoters examined. In addition, photochemical cross-linking experiments indicate that TFB2 forms transcription initiation complexes that are very similar in overall orientation to those formed with TFB1. From these data we conclude that TFB2 is a functional TFB-related transcription factor, and that it is likely to function as a transcription initiation factor when it is expressed in vivo.

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CLONING AND USE AS A MOLECULAR TOOL OF THE PHYTOENE DEHYDROGENASE GENE FROM THE CAROTENOID PRODUCING THERMOPHILE, *THERMUS FLAVUS*

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Thermus sp. are gram-negative aerobic thermophilic microorganisms that grow at temperatures between 50°C and 82°*C* . *Thermus* strains produce carotenoids with phyotene and delta carotene as intermediates, and various thermozeaxanthins as the end products of the carotenoid biosynthetic pathway of *T. thermophilus*. The carotenoid pathway of *Thermus thermophilus* HB27 was shown to reside on a 250 kb plasmid. Bacterial carotenoid biosynthesis schemes generally convert geranyl pyrophosphate to farnesyl pyrophosphate to geranylgeranyl pyrophosphate to phytoene to lycopene to ß-carotene. Phytoene dehydrogenase catalyses the conversion of phytoene to lycopene. We describe the isolation of a 1620 bp DNA sequence (GenBank accession # AF533751) that encodes for the 539 amino acid phytoene dehyrogenase protein of *Thermus flavus*. The deduced amino acid sequence of this protein showed 58% or less similarity to other phytoene dehydrogenases/desaturases. Phytoene dehydrogenase activity was confirmed by phytoene accumulation in *T. flavus* and *T. thermophilus* strains where the 1620 bp DNA fragment was interrupted using a thermostable kanamycin nucleotidyl transferease gene (Km^R) . Because *phyD*$^-$ colonies are recognized by a white colony phenotype, and wildtype phenotype is restored by transformation with DNA containing an intact *T. flavus phyD* gene, this provides a screening tool for the introduction of genetic information into *Thermus sp*. The phyD from *T. flavus* is the first thermostable phytoene dehydrogenase described and can function at temperatures up to 82°C, and possibly higher.

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CHARACTERIZATION OF DNA POLYMERASE I FROM *THERMUS* SP. STRAIN KW11

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A gene of DNA polymerase I (Pol I) of *Thermus* sp. strain KW11 was cloned, sequenced, and expressed in *E. coli*. The strain KW11 was isolated from a hot springs located in the Gunma, Japan, and was considered to be a new species of the genus *Thermus* based on its properties and phylogenetic relationships among the members of the genus *Thermus*. KW11 Pol I was encoded by 2502 bp (833 aa) and its putative molecular mass was 94 kDa. The southern hybridization experiment indicated that the gene of Pol I was presented in the genome of KW11 as a single copy gene. The amino acid sequence of KW11 Pol I showed identities of 89.8%, 86.9%, 86.4%, and 78.5% with the ones from *T. aquaticus*, *T. thermophillus*, "*T. flavus*", and *T. filiformis*, respectively. Six highly conserved sequence motifs; FNxxSxxQL (Pol-motif I), ILxxRxxxKL (Pol-motif IIa), TxTGRL (Pol-motif IIb), DYxQxE (Pol-motif III), AKxxxxGxxYG (Pol-motif IV), VHD (Pol-motif V), which are generally found in the primary structures of bacterial Pol I, were also found in KW11 Pol I. The KW11 Pol I was successfully expressed in *E. coli* BL21 (DE3) cells, and was purified by heat treatment and DEAE-sepharose column chromatography. The purified enzyme showed high specific DNA polymerase activity at 70 degrees C, pH 7.0. Other properties of KW11 Pol I will be discussed in detail.

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PROTEOME ANALYSIS OF FACULTATIVE ALKALIPHILIC *BACILLUS HALODURANS* C-125

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Bacillus halodurans C-125 is one of best-characterized alkaliphiles and the whole-genome sequencing project has been successfully completed by our group. To investigate the production pattern of proteins under neutral and alkaline pH conditions, total and membrane protein fractions of the *B. halodurans* C-125 cells obtained from pH7.0- and pH10.0-cultuer were analyzed by two-dimensional gel electrophoresis and nanoelectrospray mass spectrometry. The proteins extracted from different pH conditions were mainly distributed in the range of *pI* 4-7 and their molecular mass was ~ 130 kDa. The proteins, which showed the stranger expression in alkaline or neutral condition, were identified by sequencing of N-terminus and LC/MS/MS. Approximately 310 protein spots could be resolved by a gel stained with Coomasie Brilliant Blue R-250. The protein amounts of the15 spots from the pH 10-culture were twofold larger than that from the pH7.0-culture. Eight proteins that the expressions were most increased in pH10-culture were further confirmed at transcription level by northern hybridization, and the increase of the transcripts of the eight genes in the pH10-culture coincided with the increase of their translation products. When the cells of neutral culture was transferred to the alkaline medium for alkaline shock experiment, several gene products, including alanine dehydrogenase, flagillin and a function unknown small protein, showed a quickly expression increase in a very short time. On the other hand, some heat shock proteins, stress proteins and proteases were found to be expressed stranger at pH10 than pH7.0. Thus, it become clear that chaperons and some proteases response to alkaline pH conditions. For further investigation of alkaline pH inducible proteins, the protein-protein interaction analysis is in progress.

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CLONING AND HETEROLOGOUS EXPRESSION OF TWO OPEN READING FRAMES HOMOLOGOUS TO GLUTAMATE SYNTHASE SMALL SUBUNIT FROM HYPERTHERMOPHILIC ARCHAEA *PYROCOCCUS HORIKOSHII*

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Glutamate synthase (abbreviated as GOGAT) is a key enzyme in the early stages of the assimilation of ammonia in bacteria, algae and plants. It catalyses the reductive transamidation of the amido nitrogen from glutamine to 2-oxoglutarate to form two molecules of glutamate. Glutamate synthases differ in molecular weights, subunit compositions and electron donor specificities. NADPH-dependent electron transfer occurs in the small subunit of the bacterial enzyme which is around 50 kDa. The larger monomeric NADH-dependent plant enzymes have a homologous C-terminal to the bacterial glutamate synthases. The large subunit which contains the amidotransferase domain, do not exist upstream or donstream of the small subunit-like open reading frame in *Pyrococcus horikoshii*. It has been reported that the small subunit of *Thermococcus koadakaraensis* glutamate synthase formed a homotetramer structure and it showed activity. Contradicting reports claimed that two GOGAT small subunit-like open reading frames are actually one of the subunits of sulphide dehydrogenase (*sudA*) of *Pyrococcus furiosus*. The varying regulation of the two highly homologous open reading frames in microarrays showed that these two GOGAT homologues are similar only at the sequence level. Many small subunit-like regions have also been found in the *Clostridium saccharobutylicum* genome. It seems likely that these subunits are the early forms of an electron transfer domain in archaea, which later contributed to several enzymes. To further analyse this hypothesis, two open reading frames, PH0876 and PH1873, with over 90% homology at the amino acid level to bacterial small subunits of GOGAT in *Pyrococcus horikoshii* genome, have been cloned and inserted into pET42 type of expression vectors. Expression studies are on progress. This will be followed by NAD(P)H dependent activity analysis and glutamate synthase activity.

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PROTEOMIC STUDIES OF THE EXTREME ACIDOPHILE *ACIDITHIOBACILLUS FERROOXIDANS* GROWN WITH DIFFERENT OXIDIZABLE SUBSTRATES

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Acidithiobacillus ferrooxidans is a chemolithoauthotrophic acidophilic bacterium that obtains its energy from the oxidation of ferrous iron, elemental sulfur, or partially oxidized sulfur compounds. The ability of these and other microorganisms present in their habitat to solubilize metal sulfides is successfully applied in biomining operations. To further study some of the components involved in the oxidation of metals during bioleaching process, we have performed differential expression proteomics by using two-dimensional polyacrylamide gel electrophoresis of total cell proteins and periplasmic fractions of *A. ferrooxidans* grown in sulfur compounds and ferrous iron. We characterized a set of proteins changing their levels of expression during growth of the microorganism in metal sulfides and elemental sulfur compared with growth in ferrous iron. By determination of the N-terminal amino acid sequences of these proteins present in proteomic arrays obtained after two-dimensional polyacrylamide gel electrophoresis and by using the available preliminary genomic sequence of *A. ferrooxidans* ATCC 23270 we identified several of them. Genomic contexts around several of those genes suggest their involvement in the sulfur metabolism of this microorganism. Two groups of proteins could be distinguished: proteins highly upregulated by growth in sulfur compounds (and downregulated by growth in ferrous iron): a 44 kDa outer membrane protein, and exported 21kDa putative thiosulfate sulfur transferase protein, a 33 kDa putative thiosulfate/

sulfate binding protein, and a putative polysaccharide export protein. The second group of proteins were those downregulated by growth in sulphur (and upregulated by growth in ferrous iron): rusticyanin, a cytochrome c552, a putative phosphate binding protein (PstS), the small and large subunits of RuBisCO, a 30 kDa putative CbbQ protein, amongst others. In general, the results suggest a separate regulation of iron and sulphur oxidation pathways which depends on the substrate being oxidized. During growth in metal sulfides containing iron, such as pyrite and chalcopyrite, proteins upregulated both in ferrous iron and sulphur compounds were synthesized, indicating that the two energy-generating pathways are simultaneously induced depending on the kind and concentration of the available oxidizable substrates.
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CLONING AND HETEROLOGOUS EXPRESSION OF TWO SUBUNITS CODING FOR GLUTAMATE SYNTHASE HOMOLOGUES FROM THERMOPHILIC ARCHAEA *METHANOCOCCUS JANNASCHII*

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Glutamate synthase (abbreviated as GOGAT) is a key enzyme in the early stages of the assimilation of ammonia in bacteria, algae and plants. It catalyses the reductive transamidation of the amido nitrogen from glutamine to 2-oxoglutarate to form two molecules of glutamate. Glutamate synthases differ in molecular weights, subunit compositions and electron donor specificities. The ferredoxin-dependent glutamate synthases (EC1.4.7.1) of oxygenic phototrophs are monomeric proteins with molecular weights of approximately 160-180 kDa. These enzymes are generally present in green tissues of plants, while the Mr 220-240 kDa NADH-dependent forms (EC1.4.1.1.14) are more abundant in non-photosynthetic tissues such as roots, shoots and nodules. However, bacterial glutamate synthases are NAD(P)H-dependent. The bacterial NADPH-dependent (EC1.4.1.13) glutamate synthases contain two different subunits, with the larger of these subunits showing considerable homology to the monomeric ferredoxin-dependent enzymes. To understand the evolution of the gene coding for glutamate synthase and to determine its electron donor specificity, three ORFs coding for the three domains of the protein has been cloned. MJ1350 which is homologous to C-terminal beta helix domain and MJ1351 which is homologous for the FMN binding domain have been successfully expressed in *Escherichia coli* in pET series of expression vectors. Studies on the subcloning and expression of the amidotransferase homologue, MJ1351.1 and the coexpression of three ORFs are on progress.

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THERMOPHILIC LIPASES FROM GEOBACILLUS ISOLATES

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Enzymes such as hydrolases play an important role in biotechnology because of their extreme versatility with respect to substrate specificity and stereoselectivity. The use of lipases as catalysts for optical isomer-specific organic reactions is often limited by unacceptably low enantioselectivities. Lipases are versatile enzymes that can provide and environmentally-friendly means of breaking down fats for environmental clean-up and the production of value-added products. They can also be used as biocatalysts in sophisticated reactions in chemistry for the pharmaceutical industry. Lipases catalyze three types of reactions: hydrolysis, esterification and acyl transfer. The hydrolytic reaction mode is frequently used for the preparation of both carboxylic acids and alcohols in, for example, the lipase-catalyzed hydrolysis of a racemic carboxylic ester. One significant application of lipases in organic chemistry is the production of optically active compounds that may be used in sophisticated procedures for the synthesis of pharmaceuticals. Lipases are useful catalysts for the preparation of a broad range of optically active compounds and labile organic compounds. As well as regiospecific and stereoselective hydrolysis, they can be used for esterification or transesterification reactions in organic solvents, such as tert-butylmethylether, toluene and heptane. Other potential industrial applications of lipases include the production of fatty acids and glycerol via hydrolysis of oils and fats, and there are opportunities for both environmental enhancement and adding value to beef tallow by its hydrolysis to fatty acids. Beef tallow and some other fats are not in a liquid form at mesophilic enzyme temperatures and clearly, efficient enzymatic processes for handling these and related by-products require suitable thermostable lipases capable of hydrolysing long-chain fatty acids. Most lipases on the market are impure and poorly characterized and importantly, operate under relatively mild conditions at low temperatures. We have described consensus primers for the isolation of lipases by Genomic Walking PCR[1] and from expression libraries[2]. The genes for thermophilic lipases from several *Geobacillus* strains and from biomass from microbial tallow hydrolysis at high temperature have been isolated and sequenced. The enzymes have had a his-tag added and they have been isolated and purified after expression in *Escherichia coli*. We shall present biochemical data on the thermophilic lipases in hydrolytic reactions and will describe their outstanding enantioselectivity following the acylation of *(R,S)-1*-phenylethanol with vinyl acetate.

[1]P. J. L. Bell, A. Sunna, M. D. Gibbs, N. C. Curach. H. Nevalainen and P. L. Bergquist. Microbiology **148:** 2283-2291, 2002
[2]P. J. L. Bell, H. Nevalainen. H. W. Morgan and P. L. Bergquist. Biotechnol. Letts. **21:** 100 -1006, 1999.

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GENOMIC ANALYSIS OF METAL METABOLISM IN *SULFOLOBUS SOLFATARICUS*

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Sulfolobus solfataricus is a hyperthermophilic and acidophilic archaeon. Acidic hot springs, the natural environment of *Sulfolobus*, are usually rich in heavy metals due to the increased solubility of minerals at high temperature. Initially, the toxicity of metal ions in hyperthermophilic microorganisms was studied because of the potential application of these organisms in bioleaching. More recently, metal resistance in hyperthermophilic archaea has increasingly attracted interest due to its potential impact on bioremediation. In this work, the complete genome sequence of *S. solfataricus* was analyzed using function prediction methods based on sequence similarity and genome context. As resulted from our analysis, the *Sulfolobus* genome encodes a large number of genes with potential roles in metal metabolism. These include sequences of putative metal ion transporters, transcription regulators, metal binding proteins, oxido-reductases. Further analysis employing genome context function prediction methods revealed the presence of systems for the uptake, homeostasis and detoxification of metals. The genomic results were supported by growth data obtained by culturing *S. solfataricus* on minimal medium amended with toxic metal ions (Cd^{2+}, Ni^{2+}, Cr^{6+}, Ag^{2+}, As^{3+}) and toxic levels of biologically relevant metals (Cu^{2+}, Zn^{2+}, V^{4+}, Fe^{3+}, Co^{2+}, Mn^{2+}). Experimental characterization of these putative metal resistance-related genes, by gene knock-out, DNA microarrays analysis and complementary methods, needs to be undertaken for definitive functional assignments.

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IS THE METABOLISM OF DIVERSE HYDROGENOGENIC CARBOXYDOTROPHS DETERMINED BY SPECIFIC-TYPE CARBON MONOXIDE DEHYDROGENASES AND HYDROGENASES?

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Anaerobic carbon monoxide dehydrogenases (CODHs) are phylogenetically diverged enzymes occurring in phylogenetically and physiologically diverse prokaryotes: bacteria and archaea; mesophiles and thermophiles; hydrogenotrophic and aceticlastic methanogens, acetogens, hydrogen-producing CO oxidizers. Among the latter organisms, capable of growing at the expense of the reaction $CO+H_2O=CO_2+H_2$, the primary structure is known only for CODHs of *Carboxydothermus hydrogenoformans* and *Rhodospirillum rubrum*. The genome of the thermophile *C.hydrogenoformans* contains five genes for CODHs, which are phylogenetically distinct, being scattered over the phylogenetic tree of CODH genes occurring in bacteria and archaea. Biochemical data of Svetlitchnyi et al. (2001, 2004) and Soboh et al. (2002) suggest that CODH I is involved in the enzyme complex catalyzing CO oxidation with hydrogen production, CODH II is involved in CO oxidation with NADPH production, and CODH III is involved in acetyl CoA synthesis. The functions of CODH IV and CODH V remain unknown. To determine whether the metabolism of diverse hydrogenogenic carboxydotrophs is determined by specific-type CODHs and hydrogenases, we designed primers specific for the five different CO-dehydrogenase genes of *C.hydrogenoformans* and the hydrogenase involved in its CO-oxidizing H_2-producing complex. Several collection cultures of phylogenetically diverse thermophilic hydrogenogenic carboxydotrophs gave positive reaction in PCR with primers specific for type I CODH, whereas all negative controls gave negative reaction. Neither of the hydrogenogenic carboxydotrophs other than *C.hydrogenoformans* exhibited the presence of CODH II, III, IV, and V genes. The presence of the specific-type hydrogenase gene generally correlated with the presence of the CODH I gene. The results of PCR were confirmed by sequencing of the amplification products, which placed the newly revealed CODHs of hydrogenogenic carboxydotrophs in one phylogenetic cluster with CODH I of *C.hydrogenoformans*. These results are in line with the enzymological data available for *C.hydrogenoformans* but were however rather unexpected given the phylogenetic diversity of the microorganisms that we found to harbor this enzyme. The primers specific for type I CODHs may be a rather informative tool for molecular ecological studies, since they allow detection of the occurrence of the process of hydrogenogenic carboxydotrophy driven by phylogenetically diverse organisms.

Soboh B, Linder D, Hedderich R. (2002) Eur J Biochem 269:5712-5721.
Svetlitchny, V, Peschel, C, Acker, G, Meyer, O (2001) J Bacteriol 183:5134-5144
Svetlitchnyi V, Dobbek H, Meyer-Klaucke, W, Meins T, Thiele, B, Romer, P, Huber, R, Meyer, O (2004) Proc Natl Acad Sci USA, 101:446-451.

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THE SURPRISES OF ARCHAEAL PHYLOGENY: WHAT CAN *METHANOPYRUS* TELL US ABOUT GENOME EVOLUTION?

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We have built phylogenetic trees of the archaeal domain based on the fusion of amino acid sequences from 1) ribosomal proteins and 2) RNA polymerase subunits present in all archaeal genomes sequenced to date (Matte-Tailliez O, Brochier C, Forterre P, Philippe H. *Mol Biol Evol.* 2002, 19:631-9; Brochier C, Forterre P, Gribaldo S. *Genome Biol.* 2004, 5:R17 and recent updates). These "translational" and "transcriptional" trees are completely congruent with each other for all archaeal species with one major exception, *Methanopyrus kandleri*. This archaeon is grouped with the Methanobacteriales and Methanococcales in the translation tree, whereas it branches at the base of the euryarchaea in the transcription tree (as in the rRNA tree). We think that the position of *M. kandleri* in the translation tree

is the right one because the branch leading to *M. kandleri* is especially long in the transcription tree. The high evolutionary rate of the *M. kandleri* RNA polymerase is also exemplified by the presence of an abnormally high number of indels in its large subunits. Examination of the RNA polymerase subunit composition in all Archaea whose genomes have been sequenced revealed that *M. kandleri* is the only archaeon lacking a protein annotated as the RpoM subunit. This protein corresponds in fact to the transcription factor TFS (Hausner W, Lange U, Musfeldt M. *J Biol Chem.* 2000, 275:12393-9). This factor is involved in the accuracy of transcription because it is required to free RNA polymerase from stalled complexes by inducing cleavage of the mRNA (Lange U, Hausner W. *Mol Microbiol.* 2004, 52:1133-43). We wondered whether the absence of TFS could explain the high rate of evolution of the transcriptional apparatus, and perhaps other oddities of *M. kandleri* as well. Sequencing the *M. kandleri* genome has in fact revealed that this archaeon contains an unusually high proportion of fused or split gènes (Slesarev, A et al. *Proc Natl Acad Sci U S A.* 2002, 99:4644-9), besides the already well known cases of fused histones and split reverse gyrase. *M. kandleri* also contains an unusually high proportion of orphan genes, suggesting a high frequency of gene capture and loss during its evolution. If this hypothesis is correct, it would imply a previously unnoticed link between transcription and the rate of genome evolution. There are TFS homologues in eukaryotes (TFIIS) and functional analogues in Bacteria (GreA and GreB). We have prepared an *E. coli greA greB* double mutant to test our hypothesis. We will discuss the results of preliminary analyses of its phenotype.

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COMPARATIVE GENOME ANALYSES OF THE HYPERTHERMOPHILIC CRENARCHAEA SULFOLOBUS ACIDOCALDARIUS AND HYPERTHERMUS BUTYLICUS

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The 2,225, 668 bp genome of Sulfolobus acidocaldarius, growing at 80°C and pH 3-3.5, carries 2,319 predicted genes. In contrast to the genomes of S. solfataricus and S. tokodaii no intact IS elements or MITEs were detected and there is a low level of repeat structures. About 75% of the S. acidocaldarius genes are homologous to genes present in the S. solfataricus and S. tokodaii genomes although there is evidence of extensive rearrangements having occurred throughout the genomes after the three Sulfolobus diverged. Hyperthermus butylicus is a peptide-fermenting archaeon isolated from a solfataric habitat on the sea floor off the coast of Sao Miguel, Azores [1]. With a maximal growth temperature of 108°C, H. butylicus is one of the hottest organisms whose genome has been sequenced. The 1,695 predicted genes of the 1,667,186 bp genome show the highest degree of similarity to the genes identified in Aeropyrum pernix. Together with A. pernix, H. butylicus belongs to a small group of hyperthermophilic crenarchaeota that show an extremely high fraction of non-standard start codons (37% UUG, 25% GUG). The usage of transcription and translations signals in H. butylicus (and A. pernix) differs significantly from that known in the Sulfolobales [2].

At the meeting we will present the latest results on genome analyses and genomic comparisons.

[1] Zillig, W., Holz, I., Janekovic, D., Klenk, H.-P., Imsel, E., Trent, J., Wunderl, S., Forjaz, V. H., Couthinho, R., Ferreira, T. 1990. J Bacteriol. 172: 3959-3965.

[2] Torarinsson, E., Klenk, H.-P., Garrett, R.A. 2004. Envir. Microbiol., in press

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USE OF REPETITIVE SEQUENCE BASED PCR (REP-PCR) FOR GENOTYPING ARCHAEA

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Repetitive elements are short stretches of DNA that are randomly distributed throughout the chromosomes of prokaryotes. The use of PCR primers to amplify intervening sequences of DNA between specific repetitive elements in Bacteria has become a standard method for rapidly genotyping bacterial strains and providing good resolution between multiple strains within a single species. Rapid, standardized methods for high resolution genotyping of Archaea are not widely available. We evaluated a rep-PCR kit from Spectral Genomics (formerly Bacterial Barcodes) that has been optimized for genotyping Archaea. The method involved extracting DNA from the source organisms, performing a PCR-based amplification using an archaeal primer set provided in the kit, and then running the products on an Agilent, Lab-on-a-Chip DNA analyzer. Results were analyzed and compared using web-based software from Bacterial Barcodes. Over 60 different strains representing more than 20 genera of Crenarchaeota and Euryarchaeota were analyzed. All the organisms were successfully genotyped. A focus was placed on methanogens and extreme halophiles. Thus far, 8 genera and 16 species of methanogens have been analyzed, and all yielded unique genotypes. For halophiles, 11 genera and 27 different species were analyzed, and all yielded unique genotypes. The genotype patterns for any given strain were very reproducible. A comparison of 7 different strains of Halobacterium salinarium demonstrated each strain had a unique genotype. Analysis of several unknown Halobacterium spp. also indicated each was unique, although some strains clustered together suggesting they were closely related. A comparison of 4 strains of Methanothermbacter thermautotrophicus indicated that each strain produced a unique genotype. There was little systematic inference that could be made from dendrograms comparing different strains, species, and genera of archaea based on UPGMA cluster analysis. While some closely related strains did cluster together, intermixing of methanogens and halophiles was common. Based on our results, rep-PCR was a useful tool for the genotyping and strain identification of Archaea.

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USING DNA MICROARRAYS TO STUDY PHYSIOLOGY AND GENE EXPRESSION IN HYPERTHERMOPHILES

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Hyperthermophiles are microorganisms that grow optimally at temperatures at or above 80°C, and most are classified as archaea. They are a rather diverse group with respect to their metabolic capabilities. Several genera are capable of chemoautotrophic growth but most of the hyperthermophilic archaea described so far are obligate heterotrophs. Depending on the species, either peptides or peptides and carbohydrates can serve as carbon and energy sources. Excess reducing equivalents are normally disposed of as H_2S in the presence of elemental sulfur (S°) but a few species are also able to grow in the absence of sulfur and then produce H_2. The hyperthermophilic archaeon *Pyrococcus furiosus* is an excellent model system for physiological studies as it grows on peptides as well as carbohydrates, has several well established metabolic pathways, can use both sulfur and protons as electron acceptors, and has its complete genome sequence available. The *P. furiosus* genome consists of 1.9 Mbp, and 2065 ORFs are currently annotated. In this study we constructed microarrays containing all 2065 ORFs annotated in the *P. furiosus* genome. We focused on the analysis of transcripts of these ORFs in cells grown on peptides (casein hydrolysate) and in cells grown on a carbohydrate (maltose) both in the presence of sulfur. A total of 126 ORFs showed differential expression levels of more than 5-fold between the two culture conditions, and 82 of these appeared to be part of operons, indicating substantial coordinated regulation. Most of the ORFs up-regulated in maltose-grown cells appear to be involved in maltose transport, starch degradation, amino acid metabolism, an incomplete citric acid cycle and glycolysis. Enzyme assays and work by others in general verify the results obtained from the microarray analysis. Many of the ORFs up-regulated in peptide-grown cells seem to be involved in the production of aryl and acyl acids from amino acids or encode enzymes involved in gluconeogenesis. The concentrations and types of aryl and acyl acids in spent growth media were determined, and the results were consistent with the expression data. Interestingly, all ORFs encoding enzymes in the pathways of glycolysis and gluconeogenesis appeared to be monocistronic. The most strongly regulated were those encoding the enzymes glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and fructose-1,6-bisphosphatase, suggesting that these pathways in the *P. furiosus* are very tightly regulated.

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COMPLETE GENOME SEQUENCE OF DEEP-SEA PSYCHROPHILIC AND PIEZOPHILIC BACTERIUM, SHEWANELLA VIOLACEA STRAIN DSS12 AND GENOMIC SEQUENCE COMPARISON WITH MESOPHILIC S. ONEIDENSIS

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Shewanella violacea strain DSS12 is a bacterium adapting at high pressure and low temperature environments. This extremophile, a moderately piezophilic and psychrophilic deep-sea bacterium isolated from the Ryukyu trench (depth: 5,110 m), grows optimally at 30 MPa and 8 °C, but also at 0.1 MPa (atmospheric pressure) and 8 °C. It is, therefore, a useful model bacterium for comparison of various features of bacterial physiology such as mechanisms of cell division and gene expression under high and low pressure conditions. It is also valuable microorganism producing several useful enzymes (e.g. psychrophilic proteases) and bioactive substance (EPA). In addition, knowledge of the complete nucleotide sequence of the S. violacea genome, will definitely help us understanding in piezophily and psychrophily of microorganisms. From this research background, the 4,962,103 bp genome of strain DSS12 was completed in a project involving "Japan consortium for genomics of environmental microbes". In this study, we describe the whole genome analysis of piezophilic and psychrophilic deep-sea bacterium, S. violacea strain DSS12. Complete digestion of the chromosomal DNA of the strain with three rare cut restriction endonucleases, AscI, NotI and I-CeuI, showed that the total genome size was estimated as 4.8Mb. Twelve rrn operons were identified by PFGE analysis of the DSS12 chromosome digested by I-CeuI. A complete physical map of the strain was also constructed by the AscI and NotI, by means of hybridization experiments using AscI- and NotI-linking clones. Small and medium DNA inserts (2-3 and 4-5 kb) shotgun libraries in pCR-Blunt or pUC19 cloning vectors were prepared. Total of 77,711 sequences corresponding to ~8 x coverage were assembled by using Phred/Phrap/Consed software (www.phrap.org). For identification of protein coding genes, both approaches, translation to generate potential protein products of ORFs and Genaris and GenomeGambler software, indicating that 4,392 ORFs from the genome of the strain were estimated. Annotation of the ORFs in the genome showed that nearly 70% of ORFs was hypothetical or unassigned, suggesting the special mechanism of adaptation to high pressure and low temperature environments. Moreover, several industrially useful enzymes such as haloalkane dehalogenase, extracellular protease, chitinase and cellulase, were also identified. Not only comparative genomics between piezophile and non-piezophile, or psychrophile and non-psychrophile, but also applications of this bacterium in terms of biotechnology and bioremediation, will be discussed.

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USING GENOMIPHI DNA AMPLIFICATION KIT FOR MAKING WHOLE GENOME LIBRARY AND ISOLATION OF VARIOUS MIROBIAL GENES

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For roughly the last two decades, the polymerase chain reaction has been used in establishing microbial phylogenetic trees via 16srRNA characterization. This enables culture-independent methods for assessing the diversity and relative abundance of microbial populations. Prior to PCR, identifying phyla based on morphology was extremely difficult and necessarily selective. Culturing methods fail to represent the majority of microbes present in naturally occurring communities. PCR has some significant limitations. Large samples of soil, water, etc. must be subjected to DNA extraction in order to provide sufficient material to begin PCR analysis of the metagenome. Each PCR analysis is conducted from primary source material, which may be precious. Lastly, PCR generates small fragments of DNA, the sequence of which is limited *a priori* by primer design. A DNA amplification method has been developed that generates high molecular weight, representative DNA. GenomiPhi™ DNA Amplification Kit enables metagenome access using significantly less sample than traditional PCR-based methods, and the genetic material generated by this process is renewable in the lab, eliminating the need to take additional field samples. The method works by random primed amplification by Phi29 DNA polymerase. Amplification of 10^3-10^4 fold is accomplished overnight. Our research takes 6 different species, from the culture collection and directly amplifies them with the GenomiPhi method. Amplified product is either used to make whole genome library or is used directly in PCR to amplify 16sRNA PCR fragment. Both genomic library and resulting PCR products are sequenced. PCR products are analyzed against rRNA database sequences for confirmation. Degenerate primers are designed and desired genes are amplified using PCR. The desired genes are then cloned into plasmid and an active protein is harvested.

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IN VIVO AND IN VITRO SELF-SPLICING OF GROUP II INTRON WITHIN THE HOUSEKEEPING GENE, RECA OF THE THERMOPHILIC BACTERIUM GEOBACILLUS KAUSTOPHILUS

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Group II introns are catalytic RNAs that function as mobile genetic elements by inserting directly into target sites in double-stranded DNA. They are found in bacteria, mitochondria, and chloroplast genomes. Group II introns posses a consensus secondary structure comprised of six domains with specific roles, like catalysis of splicing, and expression and folding of protein. Group II introns have been found to be widely spread in bacterial species. Although it is known that some of them are inserted in the genes with unknown function, there is no example to be inserted in housekeeping gene. We discovered the group II intron, which interrupted the recA gene in the genome of thermophilic Geobacillus kaustophilus HTA426. In Blast search analysis, it was shown that the spliced recA gene was 83% identitical to RecA protein of Bacillus. anthracis Ames. By means of RT-PCR with total RNA from G. kaustophilus, the group II intron was shown to be removable from the precursor mRNA for the recA gene product. The experiment of in vitro splicing showed the same result to that of in vivo with total RNA from G. kaustophilus, indicating that the splicing of the recA gene of G. kaustophilus occurs by a self-splicing mechanism.

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THERMAL ADAPTATION OF PROTEINS IN THE METHANOCOCCALES

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Amino acid sequences of homologous proteins from mesophiles (Methanococcus maripaludis, M. vannielii, and M. voltae), a moderate thermophile (Methanothermococcus thermolithotrophicus), and a hyperthermophile (Methanocaldococcus jannaschii) were compared to investigate protein thermal adaptation. For the wide range of optimal growth temperatures spanned (over 45 C), members of this archaeal group have very similar genomic G+C contents, minimizing one of the greatest known influences on protein amino acid composition. The inclusion of a moderate thermophile in the analysis allows us to ask whether the nature of protein adaptation is different in the higher and lower temperature interval. In addition to analyses of differences in amino acid usage, the analysis of complete and nearly complete genomes allowed us to investigate the frequency of each of the 380 amino acid replacements. Changes in amino acid properties (hydrophobicity, residue volume, polarity, etc.) were also considered. The locations of changes within the three-dimentional structures of the proteins were modeled based on sequence alignments with proteins with known crystal structures. Amino acid usage within each secondary structure type was analyzed. The frequency of alpha-helix stabilizing factors, namely Glu-Lys pairs, Phe-Cys/Met pairs, and charge-dipole interactions were examined. The solvent accessibility of each residue was also calculated from the structural models. Amino acid frequencies and changes were also compared between surface and interior residues. We conclude that thermal adaptation results from changes in overall protein character that are contributed by numerous "conservative" amino acid replacements. The amino acid changes were not significantly different among the different secondary structure types or between surface and interior residues. General trends in amino acid mutations were consistent between the two considered temperature intervals, but changes in hydrophobicity and residue volume were more pronounced at higher temperatures, while changes in the number of charged residues were more pronounced at lower temperatures. This suggests that the importance of the various factors involved in thermal adaptation depends on the temperature interval.

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SEQUENCE AND ANALYSIS OF THE GENOME OF BACTERIOPHAGE BCJA1C ACTIVE AGAINST THE ALKALIPHILIC BACTERIUM, *BACILLUS CLARKII*

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The sequence of the first alkaliphilic bacteriophage genome has been determined. Bacteriophage BCJA1c is a member of the *Siphoviridae* family with B1 morphology. It is active against the obligately alkaliphilic bacterium, *Bacillus clarkii*. It possesses and isometric head which measures 65 nm in diameter and a noncontractile tail of 195 nm length. The 41kb terminally redundant genome has a % G+C content of 41.7%. It contains 59 ORFs of which 68% encoded polypeptides that showed significant sequence similarity to proteins in the GenBank databases. In approximately 40% of the cases where homologs exist, they are to uncharacterized or hypothetical proteins. The majority of the homologs are to *Streptococcus pyogenes* prophage 370.1, with the lysis and tail fiber genes showing the closest relationship to genes in *Bacillus halodurans*. Similar to certain transposase genes, the gene for the integrase (*int*) contains a ribosome slippage site. As with coliphage lambda, the repressor (C1, 141 amino acids) and Cro (60 amino acids) homologs are arranged in adjacent opposite orientations. While 5 potential promoters face *cro*, only a single one faces *c1*. These are overlaid by two potential C1-binding sites (AGCTAATTTATTAGCT). DNA replication appears to involve a RepA protein and DEAD-like helicase while recombination may be mediated by a RecA homolog and a Holliday junction resolvase.

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COMPARATIVE GENOMICS AND DIVERSITY OF VIRUSES OF EXTREME ARCHAEAL THERMOPHILES

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The best studied of the archaeal viruses are those from the family Fuselloviridae, spindle-shaped viruses (SSVs) of Sulfolobus, an extreme thermoacidophile that grows optimally at pH 3 and 80°C. These viruses are found wherever Sulfolobus is found. Their genomes are unique and have very few homologs among other organisms or viruses. In order to understand these viruses, we have sequenced the whole genome of SSVs from Yellowstone National Park in the U.S.A. and the Kamchatka peninsula in Russia and compared them to the known SSVs from Beppu, Japan and cape Reykjanes, Iceland. Despite clear gene synteny, the ca. 16kbp double stranded DNA genomes differ strikingly from each other and about 1/4 of their genomes are completely unrelated. From this sample there is no correlation between geographical separation and sequence relatedness, each of the genomes share about 60% nucleotide identity. To gather less geographically diverse information, we sequenced the entire genome of an additional Fusellovirus isolated from hot springs near Krisovik, Iceland. The genome was more similar overall to the genome of SSV2 from Reykjanes, Iceland, 70% identical nucleotides than to SSVs from other locations. However, some of the individual ORFs were more similar to genes from viruses from Japan, Kamchatka or Yellowstone. The origins of this diversity and relatedness will be discussed.

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IDENTIFICATION OF BACTERIA-LIKE LIPOPOLYSACCHARIDE BIOSYNTHESIS GENES IN THREE SPECIES OF THE ARCHAEAL GENUS *PYROCOCCUS*

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Species of the archaeal genus *Pyrococcus* are hyperthermophiles with functional growth temperatures ranging from 85° to 102°C. In general, membrane permeability increases and structural integrity decreases with increasing temperature. Lipopolysaccharide (LPS), a major component of Gram-negative bacterial outer membranes, is used to regulate the fluidity of the outer membrane in response to changes in temperature. As temperature goes up, the number of LPS molecules in the outer membrane increases, enhancing the structural integrity of the membrane. Little is known about how archaea regulate membrane integrity. However, *Pyrococcus* species do stain Gram-negative. In this study, we examined whether species of the genus *Pyrococcus* have the genetic potential to produce a bacteria-like LPS. To investigate this hypothesis, 54 genes involved in bacterial LPS biosynthesis were BLASTed against the complete genomes of three *Pyrococcus* species, *P. abyssi*, *P. furiosus*, and *P. horikoshii*. None of these species appear to have significant homology to enough bacterial genes involved in the biosynthesis of two of the three domains of an LPS molecule, lipid A and the core oligosaccharide. *P. abyssi* and *P. horikoshii* do appear to have genes with significant homology to the majority of the bacterial genes involved in the biosynthesis of the O-polysaccharide subunit of LPS. *P. furiosus* lacks homology to several key bacterial genes, so it appears unlikely this species could generate this subunit via the same pathway as the other two *Pyrococcus* species. Further investigation *in vivo* will confirm whether *P. abyssi* and *P. horikoshii* are capable of producing a bacteria-like O-polysaccharide.

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PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF SSV2-SSVX, THE *SULFOLOBUS* HELPER AND SATELLITE VIRUS SYSTEM

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Sulfolobus islandicus REY15/4 was isolated from a hot-spring in Reykjanes, Iceland. It has been shown that this strain produces two types of SSV-like virus particles: the larger particles contain ca. 15 kb circular-covalently linked DNA (ccc-DNA) whereas the smaller particles contain 5.7 kb ccc-DNA. The genomes of both elements have been sequenced. The larger one, termed as SSV2, resembles SSV1 virus from Japan, in morphology, replication, genome size and number of open reading frames (ORFs), whereas the smaller one, named SSVx, belongs to the *Sulfolobus* pRN plasmid family. It has been shown that SSV2 functions as a helper virus for the satellite virus SSVx and facilitate its spreading in a culture. SSV2, encodes a tyrosine integrase that carries out site-specific integration of the virus into host chromosomes. Integration occurs within the downstream half of the $tRNA^{Gly}$ (CCC) gene for the natural host *Sulfolobus islandicus* REY15/4 and the foreign host *Sulfolobus solfataricus* P2, which constitutes the archaeal attachment site (*attA*). SSV2 integration is highly specific for this site and it does not occur at another site with one sequence mismatch to the *attA* site in the *S. solfataricus* P2 genome. Whereas SSV2 is stably integrated into the chromosome of its natural host, the SSV2 excision activity increases during the growth of the foreign *S. solfataricus* host. SSVx does not encode an integrase, neither does it integrate into hosts'schromosomes. Unlike SSV1, viruses production is only slightly induced by UV-irradiation and replication of SSV2 and pSSVx is strictly regulated by the growth progression of its hosts. In fact, the episomal SSV2 and SSVx were maintained about one copy per chromosome during growth, and viral replication was strongly induced as soon as the growth stopped. The copy number of SSV2 increased to a maximum within four hours with an induction fold of 40-50 on average. Environmental factors such as medium pH, growth temperature, cell density shift, heat-shock and cold-shock treatments did not contribute to the virus induction. To gain an insight into the regulation of SSVx replication, transcriptional analysis of *rep*, *copG* and *PlrA* and *orf 91*genes was performed. The expression, purification and characterisations of the correspondent proteins is underway to elucidate their functional role *in vivo.*

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PLASMIDS AND VIRUSES OF THERMOCOCCALES FROM DEEP-SEA VENTS

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Our laboratories are working on mobile genetic elements of Thermococcales (*Pyrococcus* and *Thermococcus*) isolated from deep-sea hydrothermal environments. This order, composed of anaerobic heterotrophic sufur-metabolizers, is one of the predominant groups of the hyperthermophilic microbial communities from deep-sea vents. Among a first set of new isolates obtained in our laboratories, strain GE5 which was described as a novel species (*P. abyssi*) harboured a 3.5 kb cryptic plasmid, named pGT5. This plasmid was fully sequenced, and it was demonstrated that it replicated via a rolling-circle mechanism (Erauso *et al.*, 1996, J. Bacteriol. 178). This plasmid served for the construction of a first generation of shuttle vectors. *P. abyssi* strains auxotrophic for uracil (mutated in genes *pyrE* and/or *pyrF*) were successfully transformed using a polyethylene glycol-spheroplast method by such vectors carrying either the *pyrE* and/or the *pyrF* genes of *Sulfolobus acidocaldarius* as complementation markers (Lucas et *al.*, 2002, Appl. Environ. Microbiol. 68). Latter on, about 200 *Thermococales* isolates were screened for their genetic element content and 40% were found to harbour at least one plasmid. The plasmid size ranged from 2.8 to more than 35 kb, distributed in 25 distinct RFLPs. Each plasmid type, which can be carried by several strains, was unique and usually corresponded to one specific sample with few exceptions. These plasmids copiously shared homologous regions and suggest the existence of horizontal transfer and gene shuffling. To elucidate the function of these common regions, five plasmids (of size ranging between 13 to 38 kb) were cloned in *E. coli* and their genomes are currently being sequenced. The study of viruses from Thermococcales is a pioneer work we started in 1999. We performed a systematic search on samples collected in various geographically hydrothermal sites. Virus-like particles were detected by electron microscopy in several enrichment cultures. Among the different morphotypes observed, the lemon-shaped type prevailed but rigid rods, filaments and unique pleomorphic morphologies were also detected, suggesting the existence of an unexpected large diversity among hyperthermophilic Euryarchaeota (Geslin et *al.*, 2003, Res. Microbiol. 154). In parallel, we described the first VLP isolated from an hyperthermophilic Euryarchaeota, *Pyrococcus abyssi*. This particle, named PAV1, is lemon-shaped resembling the virus SSV1 of *Sulfolobus*, the type member of the archaeal virus family *Fuselloviridae*. PAV1 maintains in its host in a stable carrier state. It contains a double-stranded circular DNA of 18 kb which is also present in high copy number in a free-form in the host (Geslin et *al.*, 2003, J. Bacteriol. 185). Its genome was completely sequenced and contained 24 ORFs. Searches for structural and functional protein motifs and domains allowed the identification of putative functions.

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A *SULFOLOBUS/E. COLI* SHUTTLE VECTOR BASED ON THE GENETIC ELEMENT PSSVX FROM *SULFOLOBUS ISLANDICUS*: TRANSFECTION, TRANSFORMATION AND SELECTION BY FUNCTIONAL COMPLEMENTATION OF A Â-GLYCOSIDASE DEFICIENCY IN *SULFOLOBUS SOLFATARICUS*

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The pSSVx genetic element from *Sulfolobus islandicus* 15/4 is a hybrid between a plasmid and a fusellovirus and is able to spread as particles when the helper SSV2 virus is present in the cells. It is able to infect a broad range of *Sulfolobus* strains, such as *Sulfolobus solfataricus* P2 but, differently from the SSV2 element, it is not able to integrate into the chromosome of the hosts (1). Infection and selection of transfectants with both the helper virus SSV2 and pSSVx element was performed on a the strain GèW of *S. solfataricus*, that grows optimally at 75-80 °C. This mutant shows a stable LacS$^-$ phenotype (2): its chromosome lacks the â-glycosidase gene (*lacS*), a widespread and general genetic marker used for transformation tests. Maintenance and spreading of both genetic elements was demonstrated in propagated coltures. Transfectants with the SSV2 virus alone of both GèW and P2 were also isolated and time-dependent induction of the copy number determined. A hybrid DNA plasmid pSSVrt was constructed fusing site-specifically the *E.coli* vector pUC19 with the pSSVx DNA, purified from *E. coli* transformants and transferred into SSV2-infected *S. solfataricus* by electroporation of competent cells. Coltures of the electropored cells, harvested at different growth stages, were demonstrated to propagate efficiently the artificial plasmid with no rearrangement or recombination. Moreover the copy level and spreading capability of the hybrid vector was shown to be only slightly lower when compared to the wild type pSSVx, used as a positive control in parallel transformation experiments. DNA size-dependent viability and virulence of the satellite virus was determined by inserting DNA fragments of increasing length into the polycloning site of the shuttle vector and testing the transformation and spreading efficiency of the pSSVrt-derived constructs. In order to increase the length of the DNA fragments to be carried, sequences unnecessary for *E. coli* propagation and selection were excised from the bacterial moiety of pSSVrt, producing the new plasmid pSSVmin. This minimal vector was demonstrated to be an efficient cloning vehicle for the transfer and the expression in *S. solfataricus* of the *lacS* gene, used as a genetic marker for the complementation of the *lacS*$^-$ mutation in the GèW strain.

1. Arnold, H.P. *et al.* (1999) Mol Microbiol 34, 217-26.
2. Bartolucci, S., Rossi, M., and Cannio, R. (2003) J Bacteriol 185, 3948-57.

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GLOBAL STRESS RESPONSE TO IONIZING RADIATION IN THE HYPERTHERMOPHILIC ARCHAEON PYROCOCCUS FURIOSUS

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Organisms devote a considerable amount of their resources to the repair of DNA damage with a multitude of specialized proteins, and overlapping pathways, designed to cope with all possible insults to their genetic material. In Archaea, little is known about the pathways for DNA repair, and almost nothing is known about their regulation. Our model system, Pyrococcus furiosus, is an anaerobic archaeon that grows optimally at 100°C, and its genome has been sequenced (Maeder et al., 1999). We demonstrated the presence of very efficient mechanisms for the repair of DNA double-strand breaks and oxidative damage in this archaeon (DiRuggiero et al., 1997). Ionizing radiation of P. furiosus cells at 2,500 Grays (Gy) resulted in chromosome fragmentation. Within 4 hours of incubation at 95°C, the chromosomes were reassembled, producing actively growing cells, and demonstrating highly effective homologous recombinational repair. Using functional genomics, we investigated the proteins and regulatory networks involved in the repair of lesions produced by ionizing irradiation (oxidative damages and double-strand breaks). We performed a time course analysis of mRNA expression level following exposure of P. furiosus cells to ionizing radiation using a full-genome microarray. Data analyses provided no evidence of a coordinated DNA repair response to DNA damage. This is similar to what we found with the halophilic archaeon Halobacterium NRC1 (Baliga et al., 2004), but in contrast to the SOS-repair system found in the Bacteria. We identified a cluster of 5-putative DNA-repair genes induced specifically by gamma irradiation. This novel repair system appears to be unique to thermophilic archaea and bacteria (Marakova et al., 2002). P. furiosus oxidative stress response was characterized by the induction and co-regulation of several proteins involved in the removal of reactive oxygen species and the up regulation of a NADH hydrogenase and sodium proton antiporters. Finally, we identified several transcriptional regulators and protein kinases highly regulated by gamma irradiation.

Baliga, N. S., S. J. Bjork, et al. 2004. Genome Res. May12, Epub ahead of print
DiRuggiero, J., N. Santangelo, et al. 1997. J. Bacteriol. 179: 4643-4645
Maeder, D. L., R. B. Weiss, et al. 1999. Genetics 152: 1299-1305
Makarova, K. S., L. Aravind, et al. 2002. Nucleic Acids Res. 30: 482-96

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MOLECULAR-LEVEL RESPONSE TO SOLAR RADIATION BY *HALOBACTERIUM SP.* STRAIN NRC-1

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Extreme halophiles inhabit environments marked by high salt concentrations of NaCl (18-25%), desiccation and intense solar radiation, all of which are causative agents of damage to genetic material and cellular proteins. Coordinated cellular responses in halophiles to DNA damage caused by these factors have not been described to date. We used the extremely halophilic microorganism, *Halobacterium* sp. strain NRC-1, as model system for a molecular-level study of cell survival under UV and gamma irradiation. *Halobacterium* sp. is highly resistant to exposure to radiation, with survival without loss of viability up to 110 J/m^2 of UV-C and 1000 Gy of gamma-ray. Resistance to gamma radiation is likely a by-product of adaptations of extreme halophiles to desiccating environments, which result in similar dsDNA breaks to those caused by gamma radiation. We further investigated *Halobacterium* sp. stress response to high doses of solar radiation using a combined functional genomic and genetic approach. *Halobacterium* sp. UV-response was characterized by microarray analyzing simultaneously mRNA changes for all 2400 genes following UV irradiation at 200 J/m^2. In addition to photoreactivation, three other putative repair mechanisms were identified including a methylation-directed mismatch repair, four oxidative damage repair enzymes, and two proteases for eliminating damaged proteins. Finally, a UV-induced down regulation of many important metabolic functions was observed during repair, and seems to be a phenomenon shared by all three domains of life. Microarray analysis of mRNA changes following 2500 Gy of gamma radiation was also carried out. The results showed a global response to radiation-induced cellular damage, and open the door for further investigations into specific DNA repair pathways, and their regulation, in *Halobacterium* sp. strain NRC-1 following exposure to solar radiation.

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DNA REPAIR IN ARCHAEA: THE MISMATCH REPAIR STORY

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DNA mismatch repair (MMR) plays a key role in the recognition and repair of errors made during replication. The pathway is methylation-dependant, and the key proteins, MutS and MutL, are conserved from Bacteria to Eukarya. Comparative sequence analyses reveal that none of the archaeal genomes sequenced so far, with the exception of Halobacterium sp. strain NRC-1 and Methanobacterium authotrophicum, have homologs of those proteins. The MMR genes found in Halobacterium NRC1 are the canonical bacterial mutS and mutL genes, suggesting that it is the result of a lateral gene transfer event. However, it is likely that if an archaeal-type mismatch repair system exists, it is also be present in Halobacterium. We determined the genomic mutation rate in Halobacterium NRC1 using 5-Fluorouracil based on the rate of forward mutation at the upp gene. The upp gene encodes the enzyme uracil phosphoribosyltransferase that converts uracil to UMP in the pyrimidine salvage pathway. We found that the genomic mutation rate for Halobacterium NRC1 is similar to that of other DNA-based microorganisms, including the hyperthermophilic archaeon Sulfolobus acidocaldarius (Grogan et al., 2001). Using a functional genomic approach, we identified a d(CTAG) methylase (zim). Its expression level was significantly reduced following UV irradiation, suggesting a transient under-methylation of the DNA, and the initiation of a novel methylation-directed MMR pathway (Baliga et al., 2004). To further investigate MMR, we constructed strains of Halobacterium NRC1 with single (zim or mutL) and double (zim and mutL) in frame deletions. Mevilonin was used as a selective marker and fluctuations tests were carried out with the mutant strains, with and without prior exposure to UV irradiation, to determine their relative mutation rates.

Baliga NS et al. 2004. Genome Research May12, Epub ahead of print

Grogan, DW et al. 2001. PNAS 98.14:7928-7933

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FUNCTIONS OF CRYPTIC VIRAL ORFS IN FUSELLOVIRIDAE

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The archaeon genus *Sulfolobus* one of the largest and most ubiquitous taxa in the Crenarchaea. These extremely thermophilic and acidophilic organisms have been found around the world in Japan, Russia, Iceland, and in the Americas. The SSV1 viruses in the family *Fuselloviridae* infect Sulfolobales and is among the best-characterized viruses in the Crenarchaea. Despite this, little is known about its replication, genome integration, or even its method of entry into the host. Because most of the Open Reading Frames (ORFs) show no similarity to known genes in the public database, we are developing a system to produce knockouts and mutants for functional analysis of all of the 34 ORFs in the genome. Due to the lack of selectable markers in Sulfolobales, we are using a shuttle vector containing a bacterial plasmid and the SSV1 genome.[1] This allows us to grow the as a plasmid in *E.coli* or as a functional virus in its host. We amplify the 18.5 kbp shuttle vector using primers that will introduce specific point mutations or remove individual ORFs. This long PCR was accomplished using a high fidelity, highly processive Phusion™ polymerase from MJ Research. We removed the only gene that shows significant similarity to known proteins, the integrase gene. This gene is thought to facilitate insertion and removal of the viral genome in a specific site in the host. Constructs are being used to test specificity of the recognition of the integrase. Integrase genes from other viruses in the family *Fuselloviridae* that have different integration sites are also being tested in the recombinant construct. This research will provide us with a better understanding of the role of the integrase in the replication of thermophilic viruses and will serve as a model to elucidate the function of other ORFs in the SSV1 genome.

1. Stedman, K., et al., Genetics, 1999. **152**(4)

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ANALYSIS OF INTERRUPTED GENES IN ARCHAEA

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Sequenced genomes often revealed interrupted genes that are generally considered the result of sequencing mistakes or evolutive remnants with low or no biological importance (pseudogenes). However, recent and more detailed investigations challenged the popular belief that pseudogenes are just "junk" DNA, demonstrating that they often exhibit functional roles [Balakirev and Ayala 2003]. In addition, in the genome of the recently discovered symbiotic archaeon Nanoarchaeum equitans split genes yielding proteins active in native conditions have been identified in an unusual high number [Waters et al. 2003]. We have recently showed that an interrupted gene from the hyperthermophilic Archaeon Sulfolobus solfataricus, split by a -1 frameshift in two open reading frames (ORF) encoding for the N- and the C-terminal part of a a-fucosidase, can be converted to a single translational frame producing a fully functional enzyme by a point mutation predicted by programmed -1 frameshifting mechanism [Cobucci-Ponzano et al. 2003]. In this translational mechanism of regulation, translating ribosomes are intentionally directed, at a specific site, to shift toward -1 reading frames [Farabaugh 1996; Baranov et al. 2002]. Here, we describe an analysis on Archaeal genomes revealing so far unknown interrupted genes. Remarkably, we found that a minority of these genes has interrupted homologues in distantly related species of Archaea and that the position of the disruption is conserved, suggesting that split genes are conserved during evolution. Moreover, we show that a full-length form of the a-fucosidase is present in S. solfataricus cellular extracts, suggesting that it could be functionally expressed by a mechanism of programmed -1 frameshifting in vivo. With this in mind, re-interpretation of existing archaeal genome sequences may identify many more functional genes.

Balakirev ES, Ayala FJ. Annu Rev Genet. 2003, 37, 123-151.
Baranov, P.V. et al. Gene 2002, 286, 187-201.
Cobucci-Ponzano B. et al. J. Biol. Chem. 2003, 278, 14622-14631
Farabaugh, P.J. Annu. Rev. Genet. 1996 30, 507-528.
Waters et al. Proc Natl Acad Sci U S A. 2003, 100, 12984-12988.

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DISCOVERY OF NOVEL MICROORGANISMS FROM EXTREME ENVIRONMENT BY GENOMIC APPROACH

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It was indicated by microscopic observation or comparison of 16S rDNA sequence that many extremophiles were surviving in many hydrothermal environments. But it is generally said that over 99 % of total microbes are now uncultivable. Thus, we planned to discover uncultivable microbes through direct sequencing of DNA prepared from the extreme environments. At first, shotgun plasmid libraries were directly constructed with the DNA molecules prepared from mixed microbes in the low-temperature hydrothermal vent water at the site RM24 in the Southern East Pacific Rise (S-EPR). The entire sequences of 27 independent plasmid clones were determined, total reading length was approximate 104 kbp. It was found that from over 50% of all clones no ORF was assigned. On these clones stop codon was frequently identified in all six frames. This feature is very similar to the feature for introns or intergenic region of Eukaryote. Also some clones contained the tandem repetitive sequence of tetra nucleotides, which was previously isolated at the 5' region of some familiar disease. Some genes for bifunctional DNA polymerase from bacteriophage were recognized. The results indicated that many microorganisms with eukaryotic feature were dominant in low temperature water of S-EPR. Secondly, shotgun plasmid libraries were constructed from the environmental DNA prepared from Beppu hot springs. The entire sequences of 55 plasmid clones were determined, total reading length was approximate 274 kbp. The ORFs were easily identified from the nucleotide sequence of all clones. Thus it can be said that hot springs is good resources for searching novel genes and microorganisms. Among all ORFs identified, full length of genes encoding the Glu-tRNA synthetase and Asp-tRNA synthetase were identified. As aminoacyl-tRNA synthetase is necessary for all organisms, it was used for phylogenetic analysis. The result of phylogenetic analysis indicated the evidence that unknown and novel archaea and eubacteria are present in Beppu hot springs. At last, the mixed microbes, isolated from the hydrothermal vent water on Suiyo seamount, were used for extraction of DNA. This DNA was used for construction of shotgun library. The entire sequences of 50 plasmid clones were determined, total reading length was approximate 126 kbp. Genes encoding Pro-tRNA synthetase and Ser-tRNA synthetase were identified and also used for phylogenetic analysis. The results indicated that novel and previously unidentified microorganisms should be present in the vent water on the Suiyo seamount. My work indicates that environmental genomics, direct cloning and sequencing of environmental DNA, is powerful approach to collect novel uncultivable microbes or novel genes.

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A THERMOSTABLE PHOSPHOTRIESTERASE FROM THE ARCHAEON SULFOLOBUS SOLFATARICUS: CLONING, OVEREXPRESSION AND PROPERTIES

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Organophosphates (OPs) belong to a class of highly toxic compounds that are commonly used in the control of major insect pests and as chemical warfare agents such as sarin, soman and VX [1]. The organophosphates are toxics for all vertebrates since they irreversibly inhibit acetylcholinesterase, a key nervous system enzyme. Enzymatic detoxification of organophosphates has become the subject of many studies because alternative methods of removing organophosphate residues are impractical due to high costs or environmental concerns [2]. For this application bacterial OPs hydrolases are more interesting due to their broader substrate

specificity and higher catalytic rates. A new gene from the hyperthermophilic archaeon Sulfolobus solfataricus MT4, reported to show sequence identity with the phosphotriesterase-related protein family (PHP), was cloned by means of the polymerase chain reaction from the S. solfataricus genomic DNA. In order to analyse the biochemical properties of this protein an overexpression system in Escherichia coli was established. The recombinant protein, expressed in soluble form at 5 mg/L of E. coli culture, was purified to homogeneity and characterized. In contrast with its mesophilic E. coli counterpart that was devoid of any tested activity, the S. solfataricus enzyme was demonstrated to have a low but detectable paraoxonase activity. This activity was dependent from metal cations with Co2+, Cd2+ and Ni2+ being the most effective and was thermophilic and thermostable. The enzyme was inactivated with EDTA and o-fenantroline and activity was fully regained after metal addition [3]. A 3D homology model has been constructed and a mutagenesis approach is ongoing in order to increase the activity of the enzyme. The importance of a stable paraoxonase for detoxification of chemical wastes, chemical warfare agents and agricultural pesticides will be discussed.

1) Raushel FM (2002) Bacterial detoxification of organophosphate nerve agents. Curr. Op. Microbiol. 5, 288-295
2) LeJeune KE, Wild JR, Russell AJ. (1998) Nerve agents degraded by enzymatic foams.Nature.395, 27-8.
3) Merone,L., Mandrich, L., Rossi M. and Manco G. (2004) A Thermostable Phosphotriesterase from the Archaeon Sulfolobus solfataricus: Cloning, Overexpression and Properties (submitted)

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16S R RNA GENE, ENZYMATIC SECRETION AND CHROMIUM TOLERANCE BASED DIVERSITY AMONG THE HALOALKALIPHILIC BACTERIA ISOLATED FROM COASTAL REGION OF GUJARAT IN WESTERN INDIA

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Haloalkaliphilic bacteria (35 isolates) were isolated from 10 different habitats. Eight isolates from this study were subjected to 16S r RNA gene sequencing and results indicated varying diversity among them based sequence homology. While 3 isolates appeared new, the rest of the 8 isolates had more than 90 % sequence homology with Bacillus species, Bacillus strainVANO4, *B. psedudofirmus* and *B. haloalkalophilus*. 2 isolates, Mi1 and Si1 were 96 % similar to isolate WE1 from Lake Elmenteita, a soda lake. Interestingly, isolates Mi1 and Si1 turned out to be 100 % similar to each other on the basis of their 16S r RNA gene sequence, despite the fact that they were isolated from different and distantly apart alkaline and salt rich habitats. alkaline proteases was wide spread among the isolates, whereas only 4 secreted amylase. Chromium is the main pollutant in the effluent coming from the electroplating industries. The chemicals treatments are not that much effective to remove it completely from the effluents the process of bioaccumulation is the process of choice in this case. The eight selected isolates showed the variable degree of tolerance towards the chromium among this Bhv3 was found to be best in the primary screening up to the level of 0.1 gm% so further tolerance of Bhv3 was measured in the range of 0.1 to 0.5% % and isolate was found to tolerate the concentration up to 0.4% further analysis was done with reference to conversion of Cr^{+6} to Cr^{+4} lesser toxic form selected microorganism. This study on haloalkaliphilic bacteria from beyond soda lakes indicated the ecological significance of extracellular proteases in these organisms and this enzyme could act as a biochemical maker in judging the microbial diversity and the chromium tolerance observed here would attract biotechnological applications for bioremediation of chromium under alkalinity and high salt conditions.

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STUDY ON HYDROCARBON DEGRADATION AND BIOSURFACTANT SECRETION FROM THE ALKALOPHILIC BACTERIA ISOLATED FROM COASTAL REGION OF GUJARAT IN WESTERN INDIA

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Twenty alkalophilic bacteria were isolated from different saline and alkaline habitats from coastal region of Gujarat in Western India. Isolates were investigated for biochemical heterogeneity and hydrocarbon degradation. The isolates responded variably for utilizing crude oil, petrol, diesel, kerosene as a sole source of carbon. Selected isolates growing on crude oil as a sole source of carbon were further optimized for different physico- chemical requirements. To track the fate of the hydrocarbon further analysis was done using GC- MS, which demonstrated the degradation of long aliphatic chain to shorter ones. Apart from the hydrocarbon degrading ability the isolates were also evaluated for synthesis and secretion of biosurfactant. The wide spread presence biosurfactant secretion along with hydrocarbon degrading potential indicated the positive correlation between them. The present study on biosurfactant secretion and hydrocarbon degradation indicated the ecological significance of biosurfactant as a biochemical maker in judging the hydrocarbon degrading ability. The results indicates better prospectus for using such organisms in hydrocarbon degradation and biosurfactant production under alkaline and saline environment.

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A BETA-ENDOGLUCANASE FROM SULFOLOBUS SOLFATARICUS WHICH WORKS BEST IN HOT ACID

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The genome of the crenarchaeot *Sulfolobus solfataricus* harbours three putative â-glycosidases. In an effort to characterize thermo- and acid-stable proteins from this organism we concentrated on extracellular enzymes. The putative â-glycosidase SSO1949 has a N-terminal signal sequence suggesting an extracellular location of the protein. From sequence and structure based alignment combined

with modelling of the structure of SSO1949 we infer that the cellulase SSO1949 has a typical glycoside hydrolase fold and an active site similar to the active site of other cellulases of family 12. SSO1949 was overexpressed as a polyhistidine fusion protein in *E. coli* and purified by metal-chelate affinity chromatography and anion exchange chromatography. The purified protein degrades carboxymethylcellulose and cellooligomers larger than 4 units as revealed by thin-layer chromatography. The enzymatic properties of SSO1949 were characterized in detail by a FRET assay which uses a doubly labelled cellohexaoside as a substrate for hydrolysis. In this assay, the activity of the enzyme can be measured conveniently over a wide temperature and pH range. Surprisingly, the enzyme is most active at pH 1.8 and at 80 °C. Whereas several other thermostable â-endoglucanases have been described, no thermostable â-endoglucanases have been reported which such an extreme low pH optimum. When studying the stability of SSO1949, we found that the enzyme is not only most active but also most stable at these extreme pH conditions. For use in biotechnology, the high stability and activity of the *Sulfolobus* â-endoglucanase seems to be highly promising. The first step in the conversion of cellulose to bioethanol includes the hydrolysis of cellulose by sulphuric acid at high temperature. A thermoacidophilic cellulase as described here could help to improve the efficiency of cellulose utilization. Our data show that the extracellular â-endoglucanase SSO1949 from *Sulfolobus solfataricus* is perfectly adapted to work at high temperature and low pH, its physiological environment. The architecture of the active site seems to be unchanged with respect to "neutral" and "alkaline" â-endoglucanases of glycoside hydrolase family 12 underscoring the potential of a twenty amino acid protein world for functional adaptation to extremely harsh conditions.

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CO THRESHOLD OF CO CONVERSION TO H_2 BY *CARBOXYDOTHERMUS HYDROGENOFORMANS*

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Biological conversion of CO to H_2 may be used to produce a clean hydrogen from synthesis gas. Hydrogen gas has a wide application range. It is used in chemical synthesis, anaerobic waste water treatment, and it is considered to play a predominant role in a future hydrogen economy. Syngas is produced by partial oxidation or steam reforming of fossil fuels, biomass and waste. Main components of syngas are CO, H_2, and CO_2, but the exact composition depends on the hydrocarbon source used. CO can be converted with H_2O to H_2 and CO_2, a reaction known as the water gas shift reaction. Many applications that use H_2 are sensitive towards CO. E.g. PEM fuel cells are irreverisibly inactivated by CO. Levels below 10 ppm are required for economic operation. Current syngas refinement processes use multiple shift steps and expensive catalysts to remove CO below target values. Several bacteria are known to convert CO to H_2 according to the shift reaction. The thermophile *C. hydrogenoformans* shows rapid growth with CO as sole source of carbon and energy. H_2 and CO_2 are not used for growth. These characteristics make it the ideal model organism to study the feasability of biotechnological syngas refinement. Currently no data are available on lower CO threshold values of biological CO converting processes. Threshold studies for hydrogenotrophic processes indicate that lower H_2 thresholds are reached in reactions with lower standard Gibbs free energy changes. H_2 thresholds of 0.02 ppm are reported for nitrate reducing conditions ($4H_2 + NO_3^- + H^+ ! NH_4^+ + 3H_2O$; Ä G°' = -150 kJ/mol H_2), whereas acetogenic conditions ($4H_2 + 2HCO_3^- + H^+ ! Acetate^- + 4H_2O$; Ä G°' = -26 kJ/mol H_2) reach thresholds of only 100 ppm. Standard Gibbs free energy changes for CO conversion as performed by *C. hydrogenoformans* ($CO + H_2O ! H_2 + CO_2$; Ä G°' = -20 kJ/mol) appear limiting compared to the values for H_2. The gaseous nature of the products (H_2 and CO_2), however, improves the energetics under experimental conditions. Additionally, conditions can be adjusted to favour CO conversion. The aim of our study was to investigate minimum CO threshold that can be attained by *C. hydrogenoformans* in batch culture. The results confirm that *C. hydrogenoformans* is capable of CO removal below target values, provided that CO_2 is removed from the gasphase.

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CLONING, PURIFICATION, AND CHARACTERIZATION OF AN INTRACELLULAR GLUCOAMYLASE FROM THE THERMOACIDOPHILIC ARCHAEON PICROPHILUS TORRIDUS

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Picrophilus torridus is an extreme acidophilic Archeon, which grows aerobically on starch close to pH 0 and 60°C. The key enzyme, glucoamylase (1,4-á-D-Glucan Glucohydrolase, E.C. 3.2.1.3.) catalyses the release of single â-glucose units from the non-reducing end of starch and other related oligo- and polysaccarides. A gene (1.9 kbp) predicted to encode for an intracellular glucoamylase was cloned into Escherichia coli Rosetta™ (Novagen). Five conserved regions (I to V) typical for glucoamylases were found in the gene examined. The protein was expressed successfully and purified using heat denaturation, ion exchange chromatography, and size exclusion chromatography. Results obtained from polyacrylamide gel electrophoresis (PAGE) suggest that the native protein is an oligomer consisting of four subunits with a molecular weight (MW) of 71 kDa each. Activity of the recombinant glucoamylase was found to be highest at pH 5 and 50°C.

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CLONING AND CHARACTERIZATION OF THE ENDOGLUCANASES' COLLECTION OF THE HYPERTHERMOPHILIC ARCHAEON SULFOLOBUS SOLFATARICUS

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The hyperthermophilic archaeon Sulfolobus solfataricus grows optimally in aerobic conditions at 80°C, pH 3-4, and can utilize several carbohydrates as carbon source. The sequenced genome revealed 22 glycosidases, the highest number among the sequenced Archaea. Our group is actively involved from many years in the characterization of thermophilic exoglycosidases from this source, including a b-glycosidase, an a-xylosidase, and an a-L-fucosidase [Moracci et al. 2000; Moracci et al. 2001; Cobucci-Ponzano et al. 2003]. These intracellular enzymes are active on short oligosaccharides from xyloglucan, one of the most abundant hemicelluloses from seeds and plant cell walls, suggesting that they may be involved in the utilization of this polysaccharide as carbon and energy source, in vivo. However, the enzymatic activities involved in the degradation of xyloglucan in S. solfataricus are still unknown. Four open reading frames (ORF) in the genome of S. solfataricus belonging to glycoside hydrolase families GH5 and GH12 (http://afmb.cnrs-mrs.fr/CAZY/index.html), (SSO3007 and SSO1354, SSO1949, and SSO2534, respectively) could be involved in the degradation of polysaccharides. The expression in recombinant form and the substrate characterization of these enzymes will give insights on the functional role played in vivo and will allow their exploitation in polysaccharide hydrolysis. In an effort to characterize at enzymatic level these putative endoglucanases we describe here the cloning of the SSO3007, SSO1354, and SSO1949 ORFs and their expression in Escherichia coli. The recombinant enzyme expressed from the ORF SSO3007 showed optimal activity at 70°C on tamarind seed xyloglucan, birchwood xylan, and mannan; carboxymethyl cellulose was hydrolyzed with lower efficiency. The cooperation of this endoglucanase with the exo-glycosidases described above in the degradation of natural polysaccharides will be also discussed.

Cobucci-Ponzano et al. J Biol Chem. 2003, 278, 14622-14631.
Moracci et al. J Biol Chem. 2000, 275, 22082-22089.
Moracci et al. Methods Enzymol. 2001, 330, 201-215.

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CHARACTERIZATION OF TWO NOVEL LIPASES FROM THE ALKALITHERMOPHILE *THERMOSYNTROPHA LIPOLYTICA*

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T. lipolytica is the first obligately anaerobic thermophilic lipolytic bacteria, which hydrolyzes triglycerides and utilizes the liberated short and long chain fatty acids. Two enzymes, termed lipA and lipB, were purified to gel electrophoretic purely form from a culture grown for 24h in a 22 L fermentor at 65°C and $pH^{25°C}$ 8.0 by ammonium sulfate precipitation, octyl sepharose and Q-sepharose chromatography. The (SDS- PAGE) molecular weights of lipA and lipB are 47 and 52 KDa, respectively. The temperature optima of lipA and lipB are 100°C and 92°C, respectively, these are the highest among all known lipases so far. The $pH^{25°C}$ optima of lipA and lipB are 8.8 and 9.1, respectively. LipA is more thermostable; it was stable at 80°C for 28 h, it retained 50% of activity after 4 to 8 h incubation at 100°C, compared to 2h for lipB. Significant inhibition of activity of both lipases was observed at 10mM PMSF, suggesting that a serine residue plays a crucial rule in the catalytic mechanism.10 mM of EDTA inhibited totally the activity, suggesting the involvement of metals. However, the addition of detergent to the partially purified enzymes stimulated the activity, e.g. 1% Tween20 increases the activity by 4 and 13 folds to lipA and lipB, respectively. Both enzymes prefer glycerides with long chain fatty acids (C_{12} to C_{18}) as substrates and show low activity with C_4 (lipA 24%, lipB 15%) and C_6 (lipA 65%, lipB 45%) . Thin layer chromatography results showed that both lipases catalyze the hydrolysis of ester bonds at position 1 and 2 (2, 3).

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CYCLIC DIPEPTIDES AND ANTIBIOTIC ACTIVITY OF A BACTERIUM STRAIN FROM MARINE SEDIMENT

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Cyclic dipeptides from animals have been identified to be important to health . Cyclic dipeptides have been also isolated from marine bacteria such as *Vibrio sp*. Some bioactivities of bacterial cyclic dipeptides were identified recently . A strain of halotolerant bacterium , ZS110, was isolated from marine environment of South China Sea. Five new cyclic dipeptides were isolated from the fermentation broth of the strain and they are cyclo(Leu-Ile), cyclo(Val-Leu), cyclo(Ala- Val), cyclo(Ala-Ile) and cyclo(Ala- Leu). The cyclo(Ala-Val) from other bacteria was found to be a regulatory molecule which activates bacteria communication signal AHL . When growing in medium containing starch as the carbon source, the bacteria also produced a thermostable antibiotic compound which strongly inhibited growth of Gram-positive bacteria. The growth of ZS110 reached the stationary phase after 2 days of incubation and the maximum accumalation of antibiotic activity was also observed after 2 days of incubation . The antibiotic production decreased substantially when starch in the medium was substituted with saccharides such as glucose and sucrose. Na^+ is necessary for the growth of the strain ZS110. The tolerance concetraction of the bacterium to NaCl is about 3M, although the optimum concentraction of NaCl for the growth of the strain is about 0.2M. The strain may grow at the temperature from 15 - 55°C and the optimum temperature for the growth is 45°C. It is difficult to taxonomically identify the bacterium by its morphological and physiological characteristics . 16S rDNA sequence of the strain ZS110 was found to be highly similar (99%) to that of *Bacillus subtilis* .

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A GLYCEROL-INDUCIBLE HYPER-THERMOSTABLE LIPASE FROM *BACILLUS* SP.: FERMENTATION AND CHARACTERIZATION

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Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis of triacylglycerides into diacylglycerides, monoacylglycerides, glycerols and fatty acids. Under microaqueous conditions, they catalyze the reverse reaction of esterification. Microbial lipases have currently received considerable attention with regard to their applications in detergent formulation, fat and oil processing and chemical synthesis. Most of the industrial processes prefer thermostable lipases as they increase substrate solubility, enhance enzymatic reactions and reduce the likelihood of contamination. Besides this, they also catalyze solvent free transesterification and interesterification reactions, which are important for food industry. Here, we report the media optimization of a hyperthermostable neutral lipase from Bacillus sp. The present enzyme is stable over a wide range of pH from 2.0-12.0 with optimum activity at pH 6.0. The lipase was thermoactive in the range of 40°C-100°C with a half-life of more than 4 h at 100°C. The enzyme has novel properties as its production is glycerol-inducible and growth independent.Medium optimization involving both one-at-a-time and statistical designing led to overall increase of around 193 fold (62 U/ml) in lipase production. The signal factors affecting the production of the present lipase were identified by Plackett-Burman design. Oil was a poor inducer of the lipase with only 0.32 U/ml lipase units onbtained in oil-containing production medium. Instead, sugars and sugar-alcohols (galactose, lactose, glycerol, mannitol and sorbitol) were good inducers of the enzyme. Of these, glycerol which led to about 7 U/ml lipase production was selected. Amongst various nitrogen sources used, lipase production was best supported by inorganic nitrogen sources with higher concentration of NH_4Cl (25 g/l) drastically increasing lipase production by almost 4-fold (30 U/ml). Time kinetics of lipase production in the medium containing glycerol (10 ml/l), NH_4Cl (25 g/l) and casein (2 g/l) revealed that maximum lipase production (42 U/ml) occurred in 24 h. It was also observed that lipase induction in glycerol medium was a growth independent phenomenon and hence a higher initial inoculum density supported higher lipase production. Final optimization of the production conditions was done by Response Surface Methodology taking ammonium chloride, glycerol and inoculum density as the variables. Ammonium chloride and inoculum density were the significant factors. Maximum lipase yield (62 U/ml) was obtained with a high concentration of ammonium chloride (35 g/l) in the presence of low levels (10 ml/l) of glycerol and high inoculum density (15%). The optimized production conditions were also successfully scaled up to 200 ml medium in 1000 ml flasks leading to an enzyme production of about 60 U/ml.

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HYPERALKALOPHILIC THERMOSTABLE KERATINASE FROM *BACILLUS LICHENIFORMIS* RG1: ROLE IN FEATHER DEGRADATION

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Feather constitutes over 90% protein, the main component being â-keratin, a fibrous and insoluble structural protein extensively crossed linked by disulfide bonds. The insoluble nature of keratin makes it resistant to digestion by animal, plant and many other known microbial proteases leading to serious disposal problems. Use of keratinolytic microorganisms for feather degradation is an economic biological alternative. A feather-degrading Bacillus was isolated in our laboratory that produced a hyperalkalophilic thermostable keratinase. The enzyme was stable upto pH 10, with an optima at pH 11. Incubation of the enzyme at pH 11 overnight lead to a 2- fold increase in enzyme activity. The enzyme was also stable upto a temperature of 80°C with a T 1/2 of 20 min at this temperature. Complete degradation of feathers was achieved within 24 hrs by growing the organism in feather- peptone medium, however only 0.5% degradation was observed when the extracellular cell free broth was incubated with feather. Studies were performed to understand the biochemical basis of feather degradation. A synergistic four-fold increase in keratinolytic activity (1712 U) was observed on mixing intracellular fraction (70 U) with the extracellular broth (425 U). However, the caseinolytic activity (1254 U) remained unchanged on mixing the intracellular pool with the extracellular broth (1524 U). An increase in the extracellular sulfydryl concentration (0 -180 mM) within 24 hrs was observed indicating the involvement of disulfide bond reduction in the process. In this respect, sulfite was detected in the extracellular broth and disulfide reductases in the intracellular pool. The cell disulfide reductases were found to increase with time (0 - 54 U within 24 hrs) as feather degradation progressed indicating their role in sulfitolysis. Thus a mechanism of keratinolysis for Bacillus licheniformis RG1 is proposed where initial attack by disulfide reductases on disulfide bonds of keratin along with sulfite reduces the protein exposing the keratin structure for further degradation by keratinases. Cell free broth alone does not contribute much to feather degradation and colonization of bacteria is important for complete degradation to occur.

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HYPERTHERMOPHILIC ANAEROBIC BACTERIAL CONSORTIUM FOR MICROBIAL ENHANCED OIL RECOVERY: A POTENTIAL TECHNOLOGY EXPLORED

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Certain microorganisms survive under extreme conditions with their inherent ability to quickly adopt to such environments. The unique feature of these extremophiles has been an interesting phenomenon, which recently prompted researchers to study and eventually utilize them in various applications. In the present study a thermophillic (active at 90°C), barophilic, anaerobic bacterial consortium was developed by assemble of three thermophillic anaerobic bacterial strain for enhanced oil recovery. Among various applications of extremophiles enhanced oil recovery is high in demand. Selected thermophillic bacterial strains were produced in bulk in a anaerobic bioreactor (100 litre working volume) at 85°C. The culture of selected thermophillic, bacterial consortium was transported under anaerobic condition from laboratory to oil field, under anaerobic conditions and injected into selected oil wells alongwith the nutrients. Total 100 m^3 nutrient mixture was injected into oil well alongwith seed culture of thermophillic bacterial strains. The Energy and Resources Institute (TERI), New Delhi has developed a hyperthermophilic, barophilic and strictly anaerobic bacterial consortium (S2) for *in situ* application into oil wells for Microbial Enhanced Oil Recovery (MEOR). This bacterial consortium was injected into oil wells for enhanced oil recovery in collaboration with Institute of Reservoir Studies, Oil and Natural Gas Corporation Ltd., Ahmedabad. After 20 days of incubation the well was opened and oil recovery started. The initial water cut 90% was reduced to 57% in the first month with the increase in the oil production from 1.93 m^3 per day to 10.3 m^3 per day. In the second month the water cut of the oil well was stabilized at 80% with oil production ranging from 7.2 m^3 to 2.5 m^3 per day in the next three months. In another oil well with liquid rate of 4.4 m^3 the water cut ration decreased from 50% to 10%. The well produced 352 m^3 of oil with a net gain of 121 m^3. The oil production ranged from 1.7 m^3 to 5.0 m^3/day. When compared with an average conventional water flood recovery, the results of microbial enhanced oil recovery showed that the residual oil recovery increased 67% and 51.49% in the first and second well respectively. The cumulative oil production in the four months of MEOR runs in the selected two well was 458m^3 and 352 m^3.

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IN VITRO EVOLUTION OF MORE THERMOSTABLE PAENIBACILLUS DEXTRANASES

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We applied directed evolution technologies combined with robotic screening methods in the development of heat-stable dextranases. Our strategy for generating improved thermostable dextranase variants relies on random mutagenesis and recombination using error prone PCR and degenerate oligonucleotide gene shuffling (DOGS), respectively. The screening of evolved/shuffled dextranase mutant libraries is based on a one-step fluorometric method using microtiter plates, a microtiter plate reader and a fluorescence detector that permits automation and sensitive quantification, meeting the key features for the detection of initially small functional changes in large mutant libraries. In this assay, dextran is hydrolysed by dextranase-containing cell lysates to isomaltose-oligosaccharides and dextranase activity is measured through the release of glucose via the combined enzymic actions of á-glucosidase, glucose oxidase and horseradish peroxidase in the presence of the fluorometric reagent Amplex Red. We have established low frequency random mutagenesis conditions (error prone PCR) resulting in an average amino acid substitution of 1.4 amino acid changes per dextranase molecule with 70, 20 and 10% of recombinants showing one, two and three amino acid substitutions, respectively. We have tested/optimised the experimental parameters of the quantitative high-throughput screen. Growth of cells in microtiter plates at an orbital rotation of 260 per minute was found (i) not to be affected by reduced oxygen availability and (ii) not to result in cross-contamination of neighbouring wells. In view of the development of a one-step assay, the addition of an antibiotic cocktail (ceftriaxone/cefotaxime) as an alternative to lysozyme-mediated cell lysis was tested and found to provide a convenient method of cell lysis, eliminating the need of cell harvesting and resuspension. In addition, while centrifugation of resulting cell lysates reduced the background fluorescence, the fluorescence of noncentrifuged cell lysates proportionally increased, removing a third handling step. To overcome the problem of robotic handling of small volumes, the feasibility of performing all four enzymic reactions of the screening assay simultaneously was investigated and was found to be feasible. Exploring the kinetics of dextran hydrolysis and oxidation, the optimal volume of cell lysate that completely hydrolysed and oxidised dextran (1 - 5 nanomoles) in a linear fashion within ten minutes has been established (20 microliters). The screening temperature has been determined (59.4°C, 20 minutes). The results of current efforts directed towards a small scale test run under optimised conditions prior to the robotic screening of evolved/shuffled dextranase mutant libraries will be discussed.

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FUNCTIONAL GENOMICS APPROACHES FOR IDENTIFYING AND CHARACTERIZING CARBOHYDRATE ACTIVE ENZYMES IN THE HYPERTHERMOPHILIC BACTERIUM *THERMOTOGA MARITIMA*

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The hyperthermophilic bacterium *Thermotoga maritima* is known to grown on a variety of poly-, oligo-, and monosaccharides. In fact, its genome has the highest number of carbohydrate active enzymes per Mb of sequence among available genome sequences. While bioinformatic tools, functional genomics studies and previous work on the biochemistry of these enzymes have enabled the annotation of the *T. maritima* genome in this respect, there apparently are still more to be identified. We have been using functional genomics approaches with a full genome cDNA microarray for *T. maritima* to investigate the differential expression on various carbohydrate growth substrates. Such an approach has enabled the discovery and characterization of several enzymes involved in galactomannan hydrolysis and catabolism, including two putative enzymes previously annotated as glucanases but now known to also be galactomannanases. Efforts to screen *T. maritima* growth on carbohydrates are ongoing. Recently, we have grown *T. maritima* on polysaccharides previously undocumented as growth substrates for this organism, including chitin (â-1,4-GlcNAc) and pustulan (â-1,6-Glc). Despite having no characterized or putative chitinase or â-1,6-endo-glucanase, *T. maritima* grows to densities in excess of 10^8 cells/mL on these sugars. Transcriptional response data for growth on these sugars were analyzed by mixed effects statistical models to determine the carbohydrate active enzymes essential for polysaccharide hydrolysis, sugar uptake, and sugar utilization. Several of the enzymes involved in these pathways are being investigated biochemically to verify suspected biochemical properties, to improve genome annotation for carbohydrate active enzymes, and to evaluate specific biocatalysts for biotechnologically relevant applications. In addition to these examples, functional genomics approaches for enzyme discovery in hyperthermophiles will be discussed.

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BIOCHEMICAL AND MOLECULAR CHARCTERIZATION OF A HIGHLY ALKALINE THERMOACTIVE LIPASE FROM *BURKHOLDERIA MULTIVORANS*

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Microbial lipases have currently received considerable attention with regard to their applications in detergent formulation, fat and oil processing and chemical synthesis. Here, we report the biochemical and molecular characterization of an alkaline thermoactive lipase from *Burkholderia multivorans* which shows maximum activity at pH 11.0 and temperature 90°C. Lipase was produced in minimal medium containing palm oil and glucose as carbon sources, ammonium chloride and di-ammonium hydrogen orthophosphate as nitrogen sources and divalent cations (Ca^{2+} and Mg^{2+}) at 37°C and pH 7.0. The lipase production was optimized using a face centered central composite design involving four parameters *viz.* glucose, oil, ammonium chloride and incubation time. The model predicted maximum lipase production (16 U/ml) in presence of 0.2% (w/v) glucose, 1% (v/v) palm oil and 0.5% ammonium chloride in a period of 24 h. The enzyme was purified by methanol precipitation followed by hydrophobic interaction chromatography using octyl-sepharose CL-4B. Lipase was eluted by reducing the ionic strength of the system by using decreasing salt gradient followed by 20% (v/v) buffered isopropanol treatment. The molecular weight of the enzyme was 30 kDa as determined by SDS-PAGE. The enzyme was characterized with reference to following properties- pH, temperature, substrate specificity, and effect of metal ions and inhibitors. The enzyme was active over a wide range of pH from 6.0-12.0 with optimum activity at pH 11.0. It was highly stable in the pH range of 2.0-12.0 (>90% residual activity). The present lipase was thermoactive in the range of 37°C-90°C with optimum at 90°C. The enzyme was stable at temperatures upto 50°C for 6 hours and had a half-life of about 5 minutes at 80 and 90°C. The lipase was active on a wide range of p-nitrophenyl esters with more than two-fold activity on p-np caproate (C6) indicating its preference for short chain esters. The enzyme was stable in a variety of organic solvents and was a serine hydrolase. The gene sequence analysis of the lipase revealed 85% homology with lipA of *Burkholderia cepacia,* a well-known commercial lipase producer.

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CULTURING MICROBES FROM PETROLEUM RESERVOIRS

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Culture-based methods were used to study the diversity of cultivable microbes in production fluid from two North Sea petroleum reservoirs (temperature 70 - 80 °C). Enrichment media for fermentatives, methanogenes, sulfide-oxidizers, sulphate-reducers and acetogenes were designed. Pure isolates were obtained from only one of the reservoirs; the isolates showed dominance of *Archaeobglobus fulgidus*, *Methanococcus thermolithotrophicus*, *Thermococcus sibiricus* and *Thermosipho japonicus* as determined by 16S rDNA sequencing. Other microbes from the isolates included á-, â-, å- and ã- subdivision of Proteobacteria (*Sphingomonas*, *Stenotrophomonas*, *Halomonas meridiana*, and *Geospirillum*) and the Gram-positive bacterium *Thermoanaerobacter ethanolicus*. Even though the enrichments from the second reservoir showed a variety of organisms, it was not possible to obtain any pure isolates from these. The 16S rDNA clones from these enrichments aligned to sequences of *Thermosipho japonicus*, *Bradyrhizobium* and *Aquabacterium*. The microbial sequences aligning to the sequences from the cultures belonged to typical species or genera associated with high-temperature conditions and/or with oil contaminated sites. Thermophilic species of *Thermotogales*, *Archaeoglobus*, *Thermoanaerobacter*, *Methanococcus* and *Thermococcus* have been reported from high-temperature oil reser-

voirs. Several of these microbes are typical sulfur-utilizers, being active in desulphurization of crude oil. These microbes may be the predominant sources for H_2S generation rather than typical sulphate-reducing bacteria, and interestingly several of them were enriched in culture media designed for SRB. Several of the sequences abundant in the cultures were not found in the clone library assembled using a culture-independent approach. This is in accordance with other studies of high-temperature oil reservoirs. This suggests that several of the predominant members of the enrichment cultures (e.g. *Thermosipho*) are not the predominant member of the reservoir communities, but show fast-growing characteristics in several of the culture media. Novel methods for enrichment and cultivation of reservoir microorganisms are being implemented, aiming to simulate the reservoir conditions as far as possible regarding media composition, oil and geochemistry/lithology, temperature and pressure.

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CHARACTERIZATION OF CHAPERONES FROM THE HYPERTHERMOPHILE *METHANOCOCCUS JANNASCHII* FOR BIOTECHNOLOGY APPLICATIONS

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Through bioinformatics analysis and microarray experiments comparing gene expression at varying high pressure-temperature conditions, we have identified potentially novel molecular chaperones in the hyperthermophile *Methanococcus jannaschii*. One of these, an individual subunit paralog of the established cochaperone prefoldin, has been characterized and compared with the natural prefoldin. Thermal aggregation and refolding assays at different temperatures were used to probe the chaperone ability of this protein. Cross-linking analysis, dynamic light scattering, and circular dichroism were used to probe its oligimerization and secondary structure. Potential applications for industrial uses, including genetic engineering techniques for improving therapeutic protein production, and stabilization of enzymes in nonaqueous solvents, will be discussed.

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PRODUCTION OF ALKALINE PROTEASE FROM THERMO TOLERANT STRAIN ISOLATED FROM NATURAL HOT SPRING

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Five strains were isolated from the natural hot spring located at Tulsishyam in Gujarat western part of India. All the strains were able to grow luxuriously till 55°C temperature and able to tolerate up to 85°C. With reference to enzyme secretion three strains were able to secrete alkaline protease and amylase secretion was reported only in one strain. Selected strain was further optimized for alkaline protease productions with reference to physicochemical requirement further studied were carried out on partially purified enzyme with reference to optimum pH, temperature requirement and thermo stability.

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GENOTYPIC CHARACTERIZATION OF EXTRACELLULAR ENZYME PRODUCING THERMOPHILIC BACILLI IN THE BALÇOVA GEOTHERMAL REGION, TURKEY

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Thermophiles are organisms that are adapted to live at high temperatures. The enzymes from thermophiles find a number of commercial applications because of their thermostabilty and thermoactivity. Therefore, the isolation of thermophilic bacteria from natural sources and their identification are very important in terms of discovering new industrial enzymes. The aim this research was the isolation of industrially important extracellular enzyme producing thermophilic bacteria from Balçova Geothermal Region and their identification by a genetic approach (16S-ITS rDNA RFLP and 16S rRNA gene partial sequence analysis).Three types of geothermal samples were collected: mud, re-injection water, and samples from uncontrolled hydrothermal vents. Isolates grown at 55°C in culture media prepared in sterilized re-injection water, were screened for extracellular enzyme activity by using eight different substrates: casein, carboxymethylcellulose, pectin, polygalacturonic acid, soluble starch, Tween 20 and 80, and xylan. In total, 109 thermoaerophilic isolates were selected. All of the isolates could hydrolyse Tween 20 (100%) but not Tween 80. Soluble starch was hydrolysed by 96%, casein by 55%, xylan and carboxymethylcellulose by 9%, and pectin and polygalacturonic acid by 2% of the isolates. The isolates were grouped into 14 different homology groups by *Taq* I and *Hae* III restriction pattern analysis of 16S-ITS (internal transcribed spacer) rDNA RFLP. Each of the RFLP groups was also studied by 16S rRNA gene partial sequence analysis. Combined analysis of 16S-ITS rDNA RFLP and 16S rRNA gene partial sequence results indicated the presence of novel or existing species of *Anoxybacillus* (9 species) and *Geobacillus* (3 species). In this study 16S-ITS rDNA RFLP was applied for the first time to differentiate thermophilic bacilli. It was also the first study on thermophilic bacilli of Balcova Geothermal site.

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A THERMOSTABLE SUGAR-BINDING PROTEIN FROM THE ARCHAEON *PYROCOCCUS HORIKOSHII* AS PROBE FOR THE DEVELOPMENT OF A STABLE FLUORESCENCE BIOSENSOR FOR DIABETIC PATIENTS

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The periplasm of Gram-negative bacteria contains a large family of specific binding proteins that are essential primary receptors in transport and, in a few cases, chemotaxis. These proteins usually have a monomeric structure that folds in two main domains linked by three strands commonly referred to as the hinge region. Conformational changes involving the hinge are thought to be necessary for sugars to get in and out of the protein binding site. Differences in the structures of the ligand-bound and ligand-free proteins are essential for their proper recognition by the membrane components. This property of binding proteins makes them good candidates as biological recognition elements in the development of biosensors. In fact, in the presence of a specific ligand, these proteins undergo a large conformational change in their global structure to accommodate the ligand inside the binding site. Based on this conformational change, sensing systems for maltose and glucose were developed using their respective binding protein. However, the use of protein-based sensors depends on protocols to enhance the protein stability such as the introduction of changes in the protein amino acid composition leading to enhanced protein structural stability. An alternative method is to use naturally thermostable enzymes and proteins isolated from thermophilic microorganisms. These macromolecules have intrinsically stable structural features and they can be considered as ideal probes for the development of innovative sensing systems. The present study reports the cloning, expression and purification of a sugar-binding protein isolated from the archaeon *Pyrococcus horikoshii* (Ph-SBP). The results show that the recombinant protein is a monomer of 55 kDa that binds glucose molecules. Circular dichroism spectra show that the protein possesses a typical á/â secondary structure organization and the interaction with glucose does not modify the secondary structure content of the protein. Fluorescence spectroscopy experiments demonstrated that the recombinant protein binds glucose with a dissociation constant of about 10 mM, a concentration of sugar very close to the concentration of glucose present in the human blood. A docking simulation on the modeled structure of the protein confirms its ability to bind glucose and proposes possible modifications to improve the affinity for glucose and/or its detection. The obtained results suggest the use of the protein as a probe for a stable glucose biosensor Moreover, starting from the coordinates of the modeled 3D structure of Ph-SBP, molecular modeling studies on glucose binding were performed. Single amino acids that may represent good candidates for modifications have been identified and their possible role e.g. as targets for labelling with fluorescent probes is discussed.

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GENETIC DIVERSITY IN MODERATE HALOPHILIC EXOPOLYSACCHARIDES - PRODUCING EUBACTERIA ISOLATED FROM HYPERSALINE ENVIRONMENTS IN THE PERUVIAN ANDES

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The moderate halophilic bacteria are exopolysaccharides (EPS) producers with interesting rheological properties, present in addition inherent characteristics that facilitate their industrial production. In order to isolate EPS- producing bacteria, samples were taken from three hypersaline environments in the Peruvian Andes which were located up to 2,500 over sea level. The samples which were sowed in sea water agar contained 0.5% yeast extract and concentrations below 3.4 M NaCl forty isolates were selected since they showed mucous growth and different phenotypic features in agar MY plus NaCl 0.85M. The genetic diversity of these isolated were determined by comparing their restriction profiles from 16S rDNA which were amplified by the polymerase chain reaction and digested with CfoI, DdeI, HaeIII and RsaI. The results were analyzed by using unweighted pair group method of association of the NTSYS-PC. Eight genotypes were found and these could indicate the existence of at least eight different species of moderate halophilic EPS-producing.

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DIGESTION BY SERINE PROTEASES ENHANCES SALT-TOLERANCE OF GLUTAMINASE IN THE MARINE BACTERIUM MICROCOCCUS LUTEUS K-3

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The mechanisms of the adaptation of proteins to high salt concentrations are remained as an unsolved problem, because of the lack of information with regard to salt-tolerant or halophilic enzymes. Salt-tolerant proteins are distinguished from halophilic proteins by their high stability and activity even at low solvent salt concentrations. Salt-tolerant glutaminase (Micrococcus glutaminase, an apparent molecular mass of 48.3 kDa, intact glutaminase) from the marine bacterium Micrococcus luteus K-3 was digested using protease derived from M. luteus K-3. The glutaminase fragment was a higher salt-tolerant enzyme than the intact glutaminase. The activity and stability of the glutaminase fragment were little affected by NaCl concentrations. On the other hand, the activity and stability of the intact glutaminase decreased as the NaCl concentrations increased. The enzymological and kinetic properties of the glutaminase fragment were almost the same as those of intact glutaminase except for salt-tolerant behavior. The digestion products were a

large fragment (apparent molecular mass of 38.5 kDa, the glutaminase fragment) and small fragments (apparent molecular mass of 8 kDa). The digestion was inhibited in the presence of 1 mM phenylmethanesulfonyl fluoride, indicating that Micrococcus glutaminase may be digested by certain serine proteases in M. luteus K-3. Digestion of intact glutaminase by serine proteases including trypsin, elastase, lysyl endopeptidase and arginylendopeptidase also produced the glutaminase fragment. The N-terminus of the glutaminase fragment was the same as that of intact glutaminase. Thus, the glutaminase fragment was C-terminally truncated. The glutaminase fragment was analyzed by MALDI-TOF mass spectrometry. The experimental value matched a region of the intact glutaminase which encompasses residues 1-368. The N-termini of two small fragments were Ala394 and Ala 396, respectively. These results suggest that the intact glutaminase possesses a region that is susceptible to hydrolysis by proteases, and that it encompasses residues 369-396. The C-terminal region of the intact glutaminase might be responsible for salt-tolerance of the intact glutaminase.

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CHARACTERIZATION OF THERMOPHILIC MICROBIAL CONSORTIA ASSOCIATED WITH OIL

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Oil reservoirs harbour a variety of microorganisms including thermophilic sulfate-reducing bacteria (tSRB), nitrate-reducing bacteria (tNRB) and methanogens. Microbial communities in oil reservoirs play important roles in oil biodegradation and reservoir "souring" (the production of sulfide by SRB). Souring is costly to the oil industry due to associated problems such as accelerated corrosion and plugging, so oil companies aim to inhibit or kill SRB. Sulfate reduction can be inhibited directly via nitrite. Nitrite inhibits sulfate reduction as it is a competitive inhibitor of dissimilatory sulfite reductase the terminal enzyme in the sulfate reduction pathway. Some SRB possess a nitrite reductase which catalyzes nitrite removal, thus enabling them to overcome nitrite inhibition. Although the problem of souring in mesophilic environments has been well investigated, thermophilic environments such as those found in deep oil reservoirs have been less studied. Microorganisms present in two different tSRB enrichments from the North Sea and an oil storage tank consortium have been characterized in this work. The North Sea tSRB enrichment cultured on a combination of acetate, propionate and butyrate was effectively inhibited by 0.5 mM nitrite and the North Sea tSRB enriched on lactate only was inhibited effectively by 0.25 mM nitrite. Sequencing of 16S rDNA from the North Sea tSRB enrichment grown on acetate, propionate and butyrate indicated the presence of *Thermodesulforhabdus norvegicus*. The oil storage tank consortium had tSRB, tNRB, nitrate-reducing sulfide-oxidizing bacteria (NR-SOB) and methanogenic activity; 4 mM nitrite was required to completely inhibit sulfate reduction. Isolate,tSRB-8A was inhibited by 0.5 mM nitrite. This consortium was able to anaerobically degrade oil organics, volatile fatty acids (VFA), hexadecane or acetate to produce methane. *Garciaella nitratireducens*, three *Clostridium spp.,* and a strain of *Methanothrix thermophila* were identified by 16S rDNA sequencing. Based on a comparison with the literature the storage tank consortium appears to contain oil-degrading bacteria that produce organic acids and hydrogen, which feed the SRB and NRB. Understanding their interactions will allow more effective control of souring in the future.

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COMPARATIVE THERMOSTABILITY/ THERMOACTIVITY ANALYSIS OF CLASS II XYLOSE ISOMERASES FOR HIGH FRUCTOSE CORN SYRUP PRODUCTION AT ELEVATED TEMPERATURES

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Enzymes from hyperthermophiles represent attractive biocatalysts for applications requiring high-temperature operations. One such application is the conversion of glucose to fructose by xylose (glucose) isomerase [D-xylose ketol isomerase EC 5.3.1.5] in the production of high fructose corn syrup (HFCS). Current HFCS production utilizes immobilized XIs from mesophilic sources, mainly *Streptomyces*, which limits operating temperatures to 60ºC. Operating at this temperature minimizes heat inactivation but limits conversion to 42-45% fructose. However 55% fructose syrups are desirable for use in food and soft drinks, thus an expensive chromatographic enrichment step is required to produce the 55% syrup. A thermostable XI operating at temperatures around 95ºC could theoretically produce 55 % fructose syrup directly, eliminating the need for chromatographic fructose enrichment. Based on sequence analysis, XI's have been catagorized into two classes, I and II. The Class II enzymes contain a ~50 amino acid insert at the N-terminus that is lacking in the Class I enzymes, although the function of the insert is unknown. While the stability and activity of the Class I enzymes are very similar, the Class II enzymes vary, with the *Thermotoga neapolitana* enzyme (TNXI) being one of the most thermostable XI currently known. Currently, only class I enzymes are used in HFCS production. The class II enzymes include XIs from mesophiles, thermophiles, and hyperthermophiles. Because of XI's leading position in the immobilized enzyme market, abundant sequence, structural, and mechanistic information is available, which makes XI's an attractive model system for investigating protein stability and biocatalysis at high temperatures. Our work focuses on the examining the TNXI enzyme and its use for high temperature HFCS production. The stability of TNXI was investigated with respect to the different activating metals (Mg^{2+}, Mn^{2+}, and Co^{2+}) and compared with other Class II XIs using differential scanning calorimetry (DSC). The functional role of the N-terminal insert was examined by using PCR to vary the length of the N-terminus of TNXI. The inactivation of TNXI was also examined and found to follow non-first order deactivation kinetics, indicating a more complex inactivating process. Finally, the *Thermotoga* enzyme was immobilized and compared with a commercially available enzyme from *Streptomyces murinus* under different process conditions.

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NEW ADVANCES IN THE DETECTION OF TOXIC COMPOUNDS BY USING THERMOPHILIC ENZYME BIOSENSORS: ORGANOPHOSPHORUS DETECTION BY A HIGHLY SPECIFIC THERMOPHILIC ESTERASE

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Biosensors are acquiring use in increasingly a broader range of applications such as clinical diagnosis, biomedicine, pharmaceutical, drug, food and drink analyses, including pollution control and monitoring. In particular, monitoring of organophosphorus pesticides is of considerable importance. These pesticides represent a very large part of agricultural chemicals due to their relatively low persistence in the environment, but some of them exhibit fairly high acute toxicity. We have characterized a carboxylesterase activity (EST2) from the thermophilic bacterium A. acidocaldarius to be employed as a device for pesticides detection. In particular, this enzyme is specifically inhibited according to a first-order irreversible reaction by a single organophosphorus pesticide, the diethyl-p-nitrophenyl phosphate (paraoxon-ethyl). Being a very fast irreversible inhibition reaction, with an half-time of less than 0.5 min, we have determined the apparent kinetic constant of irreversible inhibition by continuously monitoring the substrate reaction in the presence of the inhibitor. The obtained parameters indicate an apparent rate constant (ki) of about 166.6 10-3 s-1, an apparent affinity constant of inhibition (Ki) of about 0.023 mM-1 and a ratio ki/Ki of 7243.5 mM-1 s-1. Literature comparisons seem to indicate an inhibitor specificity of EST2 20 time higher than other acetylcholinesterases. Direct measurement of paraoxon is possible by measuring at 405 nm the release of p-nitrophenol deriving from the inhibition reaction, but better results were obtained by determining the residual activity of the inhibited enzyme on its substrate p-nitrophenyl hexanoate. The high reactivity and specificity of this compound toward EST2, make it possible to identify paraoxon concentration in the range of picomoles. Paraoxon detection was also carried out by intrinsic triptophan fluorescence and by extrinsic fluorescence, using ANS. The high yield of production in a mesophilic host (E. coli) as well as the simple purification scheme mostly based on a thermoprecipitation step, the high stability towards temperature and solvents and high specificity and reactivity, also at low temperatures (30°C), make EST2 a very attractive candidate for developing a biosensor. The availability of the 3D structure would permit the future design of optimised mutants with modified specificity towards different organophosphates.

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CONTINUOUS HYDROGEN PRODUCTION BY THE HYPERTHERMOPHILIC ARCHAEON, *THERMOCOCCUS KODAKARAENSIS* KOD1

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The hydrogen (H_2) production potential of the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1 was evaluated using a complex medium based on tryptone and yeast extract. In batch culture with the presence of elemental sulfur (S^0), the strain mainly produced H_2S and CO_2 in the gas phase, along with a small amount of H_2. When sulfur was omitted, and pyruvate or starch was added in the medium, high levels of H_2 production was observed. As the level of H_2 production correlated with growth rate, we further pursued analysis in continuous cultures. At a dilution rate of 0.2 h^{-1}, a continuous H_2 production rate of 9.46 mmol L^{-1} h^{-1} and 6.70 mmol L^{-1} h^{-1} was detected in the media supplemented with pyruvate and starch, respectively. In both cases, an accumulation of acetate and alanine was found in the culture broth. When the dilution rates were elevated in the medium with pyruvate, steady-state growth of *T. kodakaraensis* cells was observed up to 0.8 h^{-1}. Under these conditions, the maximum H_2 production (per gram dry weight) was obtained, at a high level of 59.6 mmol g^{-1} h^{-1}. Based on these results, the metabolic pathway of starch and pyruvate degradation of the strain was discussed.

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GENETIC DEVELOPMENT OF CHALCOPYRITE LEACHING *SULFOLOBUS SOLFATARICUS* STRAIN JP2

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The economical extraction of copper from low grade ores has been achieved through the use of mesophilic leaching organisms, except for the ore chalcopyrite. Leaching rates of chalcopyrite are very inefficient at ambient temperatures. Greater efficiencies can be achieved using leaching hyperthermophilic acidophiles which thrive at temperatures above 70°C and low pH. In this study, organisms isolated from geothermal environments in Papua New Guinea which could leach 1% w/v chalcopyrite were identified by 16SrDNA sequences. One of these organisms identified as JP2 *Sulfolobus solfataricus*, was chosen for strain improvement and for studying the genes involved in chalcopyrite leaching due to its close relationship to the well characterized *S. solfataricus* strain P2. To improve the leaching ability of the *S. solfataricus*, JP2 organism vector systems that could introduce novel genes or upregulate gene expression were developed. For leaching operations, a stable vector system which requires no selection pressure to maintain the vector would be required. A vector containing the SSV1 integrase gene was developed to integrate into the *S. solfataricus* chromosome. To develop high copy plasmid vectors, plasmid backbones of pGT5 (*Pyrococcus abyssi*) and pDL10 (*Acidianus ambivalens*) were tested for their ability

to replicate within *S. solfataricus*. A microarray consisting of 5300 random JP2 chromosome fragments was used to compare the RNA expression and chromosome differences between the P2 and the JP2 strains. Finally antibiotic resistant markers are being developed for *S. solfatarcus* strain JP2. Current markers either do not work in this strain or work only when the organism is grown in broth and not on gelrite plates. Several strategies have been employed which include self cloning and adapting mesophilic enzymes to high temperatures.

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ENHANCED EXPRESSION OF A THERMOPHILIC BACTERIAL XYLANASE (XYNB) IN TRICHODERMA REESEI

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Heterologous expression and secretion of a thermophilic bacterial xylanase in *Trichoderma reesei* involves several factors that need addressing. For example, it is critical that the codon usage of the foreign gene to be expressed matches that of the fungal host[1]. Also, the use of an appropriate expression vector equipped with the features for proper processing is essential. These include space for signal peptidase cleavage, a proteolytic Kex-like cleavage site for secondary processing and, when necessary, a pro-fragment from a known secreted protein for proper folding[2,3]. We previously showed that high levels of mRNA were produced from a fusion of a codon-optimised, synthetic *xynB* from the extreme thermophile *Dictyoglomus thermophilum* Rt46.B1 to the CBH1 signal sequence but low levels from a *xynB* fusion to the CBH1 core-linker region[1]. Unfortunately, very little or no XynB product was found to be secreted into the culture medium (1). Thus, three new expression plasmids (pHEN54, pHEN54RQ and pHEN*xyn1pro*RQ), all adopting the CBH1 signal sequence *xynB* fusion strategy but containing the above-mentioned features for proper processing and secretion of the XynB protein, were tested. All three constructs resulted in the thermophilic XynB being secreted into the medium. Enzyme assays showed significant xylanase B enzyme activity in the culture supernatants of several transformants, but the best activities came from the ones transformed with the pHEN54RQ-*xynB* construct. Furthermore, the bacterial xylanase appears to be glycosylated in *T. reesei,* whereas it is not when expressed in bacteria. In this presentation, we discuss the effects of the above-mentioned structural features on the production of a thermostable xylanase from *D. thermophilum* in several *T. reesei* strains.

[1]Te'o, V. S. J., A. E. Cziferszky, P. L. Bergquist and K.M. H. Nevalainen. FEMS Microbiol. Lett, **190**, 13-19 (2000).
[2]de Faria, F.P., V.S.J. Te'o, P.L. Bergquist, M.O. Azevedo and K.M.H. Nevalainen et al.,. Lett. Appl. Microbiol. **34**, 119-123 (2002).
[3]Bergquist, P. L., V. J. S. Te'o, M. D. Gibbs, A. C. E. Cziferszky, F. P. de Faria, M. O. Avezado and K. M. H. Nevalainen. Prog. Biotechnol. **18**, 239-246, (2002).

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CONVERSION OF OLIGOSACCHARIDES BY IMMOBILIZED Â-XYLOSIDASE/Á-ARABINOSIDASE FROM *SULFOLOBUS SOLFATARICUS*

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Xylo-oligosaccharides are hydrolysis products deriving from xylan by the action of xylanase. They carry acetyl, methylglucuronosyl and arabinosyl side chains, and their complete degradation can be achieved by "accessory" enzymes. The final products arabinose and xylose have interesting features from an industrial point of view. Arabinose inhibits sucrose digestion while xylose can be converted into xylitol. In this context, the production at high temperature of monosaccharides from xylo-oligosaccharides could be of interest. Since a xylanase was previously isolated from *S. solfataricus* (1), our attention was directed toward the identification of other enzymes involved in xylan degradation. The gene *xarS* encoding a putative â-xylosidase/á-arabinosidase was identified on the genome sequence of *S. solfataricus* P2 (2) and cloned by PCR amplification of the specific genomic region. The gene was overexpressed in *E. coli* and the recombinant enzyme was active against *p*NPxylose and *p*NParabinose. The recombinant enzyme was immobilized on calcium alginate since this matrix is an inexpensive support. The maximum activity of the immobilized enzyme was obtained with 0.046U/g of support, and its activity was 98% of the free activity. The optimal temperature increased of 5°C (from 85 to 90°C and from 80 to 85°C for â-xylosidase and á-arabinosidase activities, respectively). The immobilized enzyme retained 50% of the initial â-xylosidase activity after 7 months at 4°C in the presence of sodium azide. The *E. coli* Rb791 cells, containing recombinant â-xylosidase/á-arabinosidase, were also entrapped in alginate. In this manner, the purification of the enzyme was avoided. Increase of thermophilicity in immobilized cells was observed. Optimal temperature increased from 85 to 100°C for â-xylosidase and from 80 to 95°C for á-arabinosidase. Preliminar trials of xylo-oligosaccharides hydrolysis were performed with free and immobilized enzyme. Qualitative analysis by TLC revealed that xylo-oligosaccharides were almost completely converted into xylose by immobilized enzyme in a lower time with respect to free enzyme.

References

1.Cannio R et al. (2004) A xylan-degrading strain of *Sulfolobus solfataricus*: isolation and characterization of the xylanase activity *Extremophiles* 8: 117-24
2. She Q et al. (2001) The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2 *Proc Natl Acad Sci U S A.* 98: 7835-40

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DETECTION OF DNA STRAND BREAKS IN MAMMALIAN CELLS USING THE RADIORESISTANT BACTERIUM PPRA PROTEIN

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For living organisms, it is essential to stably maintain and inherit DNA that carries genetic information. However, they are always facing risk of DNA damage during life in various environments. Failure in DNA damage repair can lead to the loss of genetic information by mutation, chromosome loss, or rearrangement, and thereby in some instances, the failure results in the death of cell. Especially, DNA double-strand break is the most lethal damage. Radiosensitivities of organisms vary extensively with species. In mammalian cultured cells that are comparatively sensitive to ionizing radiation, it is important to assess intracellular distribution and generative frequency of lethal DNA strand breaks induced by irradiation in order to get insight into cell's damage response and repair potential. Therefore, the development of an effective technique to analyze DNA damage response mechanism in mammalian cells is required. However, such development had been hampered by the lack of appropriate method to evaluate the number of DNA strand breaks in cells immediately after expose to ionizing radiation. The radioresistant bacterium *Deinococcus radiodurans* is characterized by its extraordinary resistance to the lethal and mutagenic effects of ionizing and UV radiation and to many other DNA damaging agents. This resistance is considered to be due to a highly proficient DNA repair capacity. We have demonstrated that the *D. radiodurans* genome encodes a novel DNA repair protein (PprA) responsible for its DNA repair proficiency. We have found that the PprA protein possesses ability to recognize DNA carrying strand breaks. In the present study, we attempted to visualize radiation-induced DNA strand breaks with PprA protein using immunofluorescence technique to elucidate the DNA damage response mechanism in mammalian cultured cells. As a result, colocalization of Cy2 and DAPI fluorescent signals was observed. This observation suggests that DNA strand breaks in the nucleus of CHO-K1 cells were effectively detected with PprA protein. The amount of DNA strand breaks (integrated density of Cy2 fluorescent signals) was increased with the radiation dose. Thus, we have been successful in development of an effective detection method to evaluate DNA strand breaks in mammalian cells using PprA protein. Thus, this method can be useful in establishing the radiation risk assessment in molecular and cellular levels. By increasing the sensitivity for the detection of DNA strand breaks, this method can be available as a biological dosimeter on the evaluation of low-dose radiation risk. This method is also technically possible to apply to the genetic toxicity test that is used for the detection of genotoxic chemicals.

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PPRA: A NOVEL PROTEIN FROM DEINOCOCCUS RADIODURANS THAT STIMULATES DNA LIGATION

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Deinococcus radiodurans possesses extraordinary resistance to the lethal and mutagenic effects of ionizing and UV radiation and many other DNA damaging agents, which is thought to be effected by a highly proficient DNA repair capacity. The most striking feature of *D. radiodurans* is its capacity for repairing ionizing radiation-induced DNA double-strand breaks (DSBs). This bacterium can mend over 100 double-strand breaks of genomic DNA during post-irradiation incubation, whereas just a few DSBs are lethal in nearly all other living organisms. The debate concerning whether *D. radiodurans* employs a DNA repair system that includes novel components has received much attention in recent years. Over forty natural mutant strains have been isolated in our investigations dealing with DNA damage repair-deficient mutants of *D. radiodurans*. Strain KH311 exhibited significant sensitivity to many DNA damaging agents such as ã-rays, mitomycin-C, UV radiation and 4, 5 k°<1ï•□, 8-trimethylpsoralen. By analyzing this mutant strain, a unique radiation-inducible gene (DRA0346, designated *pprA*) responsible for loss of radiation resistance was identified. To gain insight into the nature of the gene product (PprA) in *D. radiodurans*, the protein was purified and characterized. Investigations *in vitro* showed that PprA preferentially bound to double-stranded DNA carrying strand breaks, inhibited *Escherichia coli* exonuclease III activity, and stimulated the DNA end-joining reaction catalyzed by ATP-dependent and NAD-dependent DNA ligases. These results suggest that *D. radiodurans* has a radiation-induced nonhomologous end-joining (NHEJ) repair mechanism in which PprA plays a critical role. PprA was able to stimulate the ligation activity of T4 DNA ligase. Given that T4 DNA ligase is widely used in DNA engineering, the possibility of making use of PprA in combination with T4 DNA ligase was examined. We found that the cloning efficiency of a commercially available TA vector with T4 DNA ligase was enhanced by more than 2-fold following the addition of an appropriate concentration of PprA to the reaction mixture. Thus, the properties of PprA, that include recognition of and binding to DNA ends and the stimulation of DNA ligation, suggest its potential use as a reagent in DNA engineering.

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ELECTROSTATIC CONTRIBUTIONS TO THE STABILITY OF PROTEINS AT HIGH TEMPERATURES

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Ionizable residues are thought to contribute significantly to the stability of proteins at high temperatures. This hypothesis is based on the observation that the number of ionizable residues in proteins

from thermophilic and hyperthermophilic organisms tends to be higher than in homologous proteins from mesophilic organisms. Furthermore, in crystal structures of thermophilic and hyperthermophilic proteins, charged moieties are often arranged in bifurcated pairs and in clusters or networks less frequently found in mesophilic proteins. The magnitude of the electrostatic contributions to stability at high temperatures remains unknown owing to the serious difficulties with rigorous solution thermodynamic studies of protein stability at high temperatures. This could be addressed, in principle, with computational methods for structure-based calculations of pKa values and electrostatic energies. However, these methods have all been calibrated empirically to reproduce pH and salt sensitive thermodynamic properties of proteins at 25 oC, and none have been extended to allow systematic treatment of the effects of temperature on electrostatic interactions. Here we introduce a modified form of the solvent accessibility modified Tanford Kirkwood algorithm that treats explicitly the temperature dependence of surface Coulombic interactions. The ability of this semi-empirical algorithm to reproduce accurately many properties of surface ionizable residues at 25 oC has been demonstrated previously. The ability of this algorithm to reproduce the temperature dependence of H+ binding properties of proteins was demonstrated in this study by reproducing the H+ titration properties of sperm whale myoglobin, cytochrome C, and staphylococcal nuclease, measured potentiometrically over a range of temperatures. We will discuss the magnitude of the effects of temperature on pKa values and on coulombic contributions to stability calculated with this method, as well as the physical and structural factors that, according to this method, determine the magnitude of temperature sensitivity of electrostatic effects. This algorithm is being applied systematically to estimate the coulombic contributions to stability over a range of temperatures in cases where crystal structures of homologous proteins from mesophilic, thermophilic and hyperthermophilic organisms can be compared.

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SEROLOGICAL AND MOLECULAR CHARACTERIZATION OF LOCALLY ISOLATED ACIDOPHILIC CHEMOLITHOTROPHIC BACTERIA USED IN COAL HEAP DESULFURIZATION

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Polyclonal antisera against whole cells of locally isolated acidophilic chemolithotrophic mesophiles (strains HC-AF2 and KC-AT2) and moderate thermophiles (local strain MT13 and standard strain *Sulfobacillus thermosulfidooxidans*) used in coal heap biodesulfurization process were successfully raised in rabbits. Among these bacteria, KC-AT2 was observed to be highly immunogenic while *S. thermosulfidooxidans* was least immunogenic. The specificity of each of the developed polyclonal antisera was determined against many other acidophilic isolates (TF9, TF10, MT9, MT10, MT16, MT17) by the serological tests including agglutination reaction, fluorescent antibody labeling reaction and enzyme linked immunosorbent assay (ELISA). The polyclonal antisera against mesophilic isolates HC-AF2 and KC-AT2 were found to be more specific as they exhibited no cross reactivity with other mesophilic and moderate thermophilic acidophilic isolates. While polyclonal antisera developed for *S. thermosulfidooxidans* and MT13 strains showed cross reactivity against other isolates (MT9, MT10, MT16, MT17) of the same species. Population of these bacteria in coal heap set for biodesulfurization was determined by quantitative ELISA, which indicated that moderate thermophiles were dominated bacteria in leach liquor while in heap both mesophiles and moderate thermophiles were detected. PCR amplification of 16S rDNA of genomic DNA from HC-AF2 and KC-AT2 indicated that they belong to *At. ferrooxidans* (98%) and *At. thiooxidans* (95%), respectively. A phylogenetic tree of these bacteria was constructed by aligning 16S rDNA sequences of other acidophilic bacteria obtained from NCBI database using various softwares available at our laboratory. Integron linked genes were amplified in these bacteria using integron specific primers HS286 and HS287. Two types of DNA bands of approximately 396 bp and 220 bp were obtained in case of both, the HC-AF2 and moderate thermophiles. Similarly in case of mixed culture the bands of same size were obtained. However, in case of pure KC-AT2 no amplification product was observed after agarose gel electrophoresis with the same set of primers.

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RECOVERY OF INTEGRON ASSOCIATED GENE CASSETTES FROM BACTERIA THRIVING IN HIGH SALT HABITATS

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Different solid and liquid samples were collected from various sites in Khewra salt mine, Pakistan. Bacterial cultures were enriched from these samples by using appropriate media. Different parameters like EC, pH, salt content and analysis of Na+ and K+ was carried out. Determination of salt contents and Preliminary characterization of the bacteria was attempted on the basis of microscopic study, growth patterns on solid media and gram staining etc. Genomic DNA was recovered from the cultured bacteria and amplified by PCR using integron specific primers HS286 and HS287. The PCR amplified DNA was cloned in pGEM T-Easy vector and clones were selected randomly and sequenced commercially. Representative of each type was selected for commercial sequencing. After sequencing BLAST search was performed with programs available through the National Center for Biotechnology Information (USA) programme (www>ncbi.nlm.nih.gov) to determine nucleotide and protein matches to the world databases. Most of the genes recovered from cultured bacteria had significant matches with existing sequences in the databases. However, the genes recovered from a library of clones from the samples were novel with no identifiable ORF homologues in the databases. This technique demonstrated the usefulness of integron capture gene recovery methodologies.

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THERMOTOLERANT *STREPTOMYCES* STRAINS ISOLATED FROM HOT COMPOST SHOW PLANT GROWTH PROMOTING ACTIVITY

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Composting is the process used to transform sugarcane by-products (filter mud and pith bagasse) to produce a soil amendment for sugarcane fields. During the thermophilic stage of the composting process, temperatures of up to 85°C are reached. In greenhouse and field tests, sugarcane producers have detected both disease suppression and growth promoting properties of the whole final compost. Analysis of the microbial community present in the composting materials showed that a high proportion of the culturable microorganisms active during the thermophilic stage were actinomycetes, many of which were also present in the final product. A screening was performed for thermotolerant strains with plant growth promoting properties. Using an agar plate bioassay 370 strains with optimal temperatures for growth ranging from 45 to 55°C were screened. A total of 50 isolates showed strong plant growth promotion. Three strains identified as *Streptomyces* spp were selected for their biological potencial to promote germination, root growth, stem elongation and foliar differentiation in turfgrass and tomato seedlings. Plate tests using sterile soil demonstrated plant growth promotion under growth chamber conditions. Preliminary fermentation tests using shaken flask cultures with two different culture media formulations showed high fermentative production of plant growth substances. Ethyl acetate and chloroform extracts from culture supernatants of the most potent strain showed auxin-like effects in agar plate bioassays. Chromatographic and spectroscopic characterization of plant growth regulators is needed to determine their chemical identity.

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SUGAR METABOLISM AND IN OSMOTOLERANT YEAST STRAINS ISOLATED FROM HONEY AND MOLASSES

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Using classical enrichment culture, forty nine yeast strains were isolated from honey and industrial molasses, among other substrates. Selected strains were analyzed with respect to their tolerance to high salt and glucose concentration using an agar plate assay, and classified as low, medium or highly tolerant to either solute. The strains were also characterized with respect to their ability to selectively ferment glucose and fructose, using culture media containing both monosaccharides. The isolates were classified according to their potential of selective catabolism of glucose (glucophilic) or fructose (fructophilic) using invert blackstrap or refinery molasses as susbstrates. Aliquots were analyzed to determine the monosaccharide composition of fermentation musts, by HPLC. A small proportion of strains were highly tolerant to glucose, a characteristic which did not correlate with tolerance to salt. Most strains were found to be either slightly glucophilic or fructophilic, but only a few were strictly selective to only one monosaccharide. Selected osmotolerant strains with a pattern of differential metabolism of glucose and fructose may provide industrial applications for the production of syrups enriched in selected monosaccharides for applications in the food industry.

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RANDOM DRIFT MUTAGENESIS (RNDM), A NOVEL METHOD FOR ENZYME EVOLUTION

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Proteins have evolved to benefit the survival of an organism and so may not exhibit features essential for *in vitro* applications. They function within the complex processes that make up a living system, and hence some of their properties may be undesirable when removed from this context. Therefore, there is considerable room for improvement of properties for which natural selective pressure was never applied. Protein engineering has the potential to dramatically enhance protein performance in a wide variety of non-natural, but technologically interesting, environments. Random drift mutagenesis (RNDM) is a technique that we have developed as a front-end complement to Degenerate Oligonucleotide Gene Shuffling (DOGS)[1], giving much greater scope and flexibility in protein evolution. This procedure is applicable to any enzyme reaction in which a chromophore or fluorescent substance that is retained in the cell is generated so that selection can be made at high speed for the desired characteristic by flow cytometric sorting. Our technique allows us to determine whether a phenotype that is derived from the interaction of multiple amino acids might require the accumulation and interaction of neutral mutations (neutral in isolation), and adaptive mutations. RNDM uses iterative misincorporation mutagenesis but no screening for adaptive mutations occurs. Instead screening would only be done for retained ability (whether unchanged, improved or reduced) to catalyze the hydrolysis of a substrate. This procedure is intended to provide high speed screening of mutants for desired characteristics without tedious assay procedures and to allow a comprehensive examination of sequence space for superior mutants. It is a platform technology applicable to any protein for which there is a colorimetric or fluorescent assay combined with flow cytometric analysis. We have used *Caldicellulosiruptor saccharolyticus* Î²-glucosidase and the substrate Imagene Green (Molecular Probes) to test this procedure and have isolated mutants with improved temperature stability and temperature optimum that have resulted from combining RNDM with gene shuffling using DOGS to give superior enzymes compared to wild type.

[1]Gibbs, M. D., Nevalainen, K. M. H. and Bergquist, P. L. Gene **271:** 13-20, 2001.

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DEVELOPMENT OF OPHIOSTOMA AS A NOVEL FUNGAL EXPRESSION HOST

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The Ascomycete *Ophiostoma* spp. comprise common sap-staining fungi that invade wood via the parenchyma cells. Some albino variants of *O. flocossum* have been used as biological control agents to prevent sap-staining. Their ability to invade wood and their secretion of enzymes outside the growing mycelium in order to utilize the non-structural components of sapwood provide an excellent basis for the development of a fungal system both for the *in situ* delivery of selected gene products into wood tissue as well as their expression and production on a larger scale in liquid cultivation. We have identified highly expressed proteins (protease and glucoamylase) for the isolation of the corresponding strong promoters for heterologous gene expression and improved their overall protein secretion by repeated rounds of UV-mutagenesis. Chromosome Walking PCR[1] was performed in order to isolate the genes encoding the dominant proteins together with their regulatory sequences (promoter and terminator). The serine proteinase is similar to the subtilisin family serine proteinase previously described for *Ophiostoma floccosum*[2]. The amylase has not been previously described before and appears to be novel. Similarity to the closest match in the database, *Aspergillus kavachii* alpha-amylase, is 60 % at the amino acid level. We have identified suitable antibiotic selection markers for efficient transformation as *Ophiostoma floccosum* conidia are uninucleate and can be transformed by particle bombardment[3]. Expression vectors featuring a strong promoter and suitable termination sequences and a transformation marker gene conferring resistance to G418 are under construction. Testing of the system is being carried out using the novel amylase (*amy1*) promoter and *dsRed1-E5* gene as a reporter/heterologous gene.

[1]Morris, D., Gibbs, M.D., Chin, C.V., Koh, M.H., Wong, K.K.Y., Allison, R.W., Nelson, P.J. and Bergquist, P.L. (1998), Appl. Environ. Microbiol 64:1759-1765.
[2]Abraham, L. D. and Breuil, C. (1996). Enzyme and Microbial Technology 18: 133-140.
[3]Te'o, V. S. J., Bergquist, P. L. and Nevalainen, K. M. H. (2002) Jour. Microbiol. Methods. **51:** 393-399.

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REVERSE GYRASE IS NOT A PREREQUISITE FOR HYPERTHERMOPHILIC LIFE

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Reverse gyrase, an enzyme that positively supercoils DNA, is the only enzyme present in all hyperthermophiles, but absent in all mesophiles. The enzyme is composed of two distinct domains, one helicase-like domain and another domain that belongs to the topoisomerase type IA family. It has been pointed out that if reverse gyrase were a prerequisite for hyperthermophilic life, the origin of life could not have been a hyperthermophile, as the two separate domains must have independently evolved in less thermophilic organisms before the occurrence of reverse gyrase. In this study, we have disrupted the reverse gyrase gene from a hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. A single reverse gyrase gene, identified from genome analysis, was replaced with a *trpE* marker gene via double crossover recombination in a host strain disrupted of *trpE*. PCR and southern blot analyses were performed to confirm the absence of the reverse gyrase gene on the chromosome of the transformant. Consistent with the absence of the gene, an apparent positive supercoiling activity that was observed in the host strain was not found in the disruptant strain. Growth rates of the disruptant strain were compared with those of the host strain at various temperatures. We found that lack of reverse gyrase led to a retardation in growth, a tendency that became greater at higher temperatures. However, disruption of the reverse gyrase gene did not lead to a lethal phenotype at 90°C. The results provide experimental evidence that reverse gyrase is not a prerequisite for hyperthermophilic life, and that the structure of reverse gyrase and its presence in hyperthermophiles no longer rule out the possibility that hyperthermophiles were the first organisms to evolve.

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MICROBIAL MOLECULAR ECOLOGY OF ANTARCTIC TERRESTRIAL SOILS

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The Dry Valleys of South Victoria Land, eastern Antarctica contain some of the most extreme biotopes on Earth and have long been considered as valid Martian analogues. While there is little or no visible evidence of life in the mineral soils of the Dry Valleys, where water contents range from 0.2 - 2% wt., classical microbiological studies have demonstrated that these desert 'soils' support low microbiological populations. In comparison, nutrient- and water-rich Antarctic 'ornithogenic' soils and terrestrial aquatic habitats give vastly higher microbial counts. Here we present the first molecular phylogenetic study of prokaryote diversity in Antarctic Dry Valley mineral soils. Comparative phylogenetic analyses of samples recovered from altitudinal transects and from nutrient-supplemented Dry Valley sites indicated that local environmental factors strongly dictate microbial diversity. High altitude low water content mineral soils were dominated by cyanobacteria (*Noctoc, Phormidium*). These phylotypes are typically associated with moist soil and aquatic biotopes, leading us to suggest that soil water content (as % wt) may be a poor indicator of water availability. No cyanobacterial signals were detected in mid- and low altitude desiccated mineral soils, which were populated by cosmopolitan organisms (e.g., *Actinobacteria*) and some 'rare' phylotypes (*Rubrobacter, Stenotrophomonas*). All sites showed numerous 'uncultured' phylotypes. Rarefaction analysis of 16S clone libraries indicated that oligotrophic soil biotopes contained relatively low Bacterial species diversity. Localised nutrient-rich soils showed both higher biomass levels and species diversity, suggesting that C/N

availability is an important factor in microbial population dynamics. Archaeal diversity was apparently restricted to members of the deep branching Type 1.1b clade of the sub-Domain Crenarchaeota. As the Antarctic continent is still largely unmodified by human habitation and activity, there is considerable international interest in retaining its 'pristine' status. In support of this objective, we have investigated the contamination of Antarctic Dry Valley sites by non-indigenous micro-organisms through human intervention. PCR analysis of samples from inhabited and 'pristine' sites showed little evidence of contamination by human enteric micro-organisms. However, we have demonstrated that human 'marker' organisms were readily identifiable in sites of human activity, but that disseminated human commensals lost culture viability rapidly. Phylotypic signals were recoverable for much longer periods than culturability, suggesting that encapsulated and naked genomic DNA is stable in Dry Valley desert soils.

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GLOBIN-COUPLED SENSORS, PROTOGLOBINS, AND THE LAST UNIVERSAL COMMON ANCESTOR

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Hemoglobins are ubiquitous in *Eukarya* and *Bacteria* but, until now, have not been found in *Archaea*. A phylogenetic analysis of the recently revealed microbial family of globin-coupled heme-based sensors suggests that these sensors descended from an ancient globin-only progenitor, or a *proto*globin. We have recently reported the discovery and characterization of two protoglobins from the *Archaea*: *Ap*Pgb from the obligately aerobic hyperthermophile *Aeropyrum pernix*, and *Ma*Pgb from the strictly anaerobic methanogen *Methanosarcina acetivorans*. Both *Ap*Pgb and *Ma*Pgb bind molecular oxygen, nitric oxide, and carbon monoxide via a heme moiety, which is coordinated to the protein through the F8 histidine (histidine 120). Mutagenesis experiments coupled with our 3-D homology model of *Ap*Pgb indicate that heme is covalently bound to the apo-globin via His120. In addition, the model predicts an intramolecular disulfide bridge aiding in *Ap*Pgb thermostability. *Ap*Pgb and *Ma*Pgb are the first identified archaeal hemoglobins. They may serve several biological functions, including protection from nitrosative and oxidative stress. We postulate that these archaeal globins are the ancestors of contemporary hemoglobins.

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CHARACTERIZATION OF MICROBES ISOLATED FROM THE MARS EXPRESS ORBITER AND ASSOCIATED ASSEMBLY ENVIRONMENTS

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Recent space exploration has increased the potential for forward contamination of other planets. Suitable techniques to eradicate resistant microbes should be improved to ensure other planetary bodies remain "pristine." Fifteen spore forming bacteria were isolated from random surfaces in the Mars Express assembly facility and surface of the Mars Express Orbiter (Baikanor, Russia). Isolates were characterized by classical colony morphology techniques as well as 16S rRNA gene sequence analysis. Phylogenetic characterization revealed presence of 5 *Bacillus* species, of which approximately 60% were *B. licheniformis*. Of microbes isolated 13 of 15 maintained resistance to dessication, heat shock (80°C for 15 minutes) exposure to UV rays (0-2 kJ/m2 at 254 nm), gamma-radiation (0-1.5 Mrad Co^{60}) and H_2O_2 vapor (3 mg/L) Several strains were resistant to individual challenges but 2 of the 15 strains were resistant to multiple perturbations. The Baikanor assembly facility operates under similar conditions to that of the spacecraft assembly clean rooms at NASA centers (class 100K). Thus the domination of *B. licheniformis* in Baikonor facility was not a surprise since this organism has been repeatedly isolated from various NASA spacecraft assembly and preparation facilities. Given the resistive nature of these spores, more strict sterilization techniques may be necessary to prevent cross-contamination.

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'*THERMOSEDIMENTIBACTER OCEANENSIS*', A NOVEL ANAEROBIC THERMOPHILIC BACTERIUM FROM OCEAN SEDIMENT CORES COLLECTED AT THE PERU MARGIN

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Background: During Ocean Drilling Program (ODP) Leg 201, deep-sea sediments at a series of sites in the eastern equatorial Pacific, the Peru Basin, and the Peru Margin were recovered. To determine whether thermophilic microorganisms can survive at suboptimal temperatures in marine sediments over long periods of time, an attempt was made to isolate thermophilic anaerobes from sediment samples collected at various depths at the equatorial Pacific sites and at the Peru Margin sites. **Methods:** Sediment core samples were inoculated in various media and incubated at 60 °C and 80 °C. Obtained isolates were characterized in respect to growth ranges and substrate spectrum and classified according to their 16S rDNA sequence. **Results:** Ten isolates were obtained from the 60 °C incubations. Three isolates came from the sediment 426 m below

sea level with a surface temperature of 9 °C (Site 1227). One from 252 m below the sea level with a surface temperature of 12 °C (Site 1228), and six isolated under sulfate-reducing condition came from the lower slope of the Peru Trench (Site 1230). All the isolates came from the inner part of core samples from the upper 1-9 m depth in the upper sediment. Based on the 16S rDNA sequence analysis, these isolates represent a novel group with *Thermovenabulum* and *Thermoanaerobacter* as their closest relatives. The cells were straight to slightly curved, terminal endospore-forming rods, 0.3-0.5 mm in diameter and 2.0-10.0 ìm in length. The temperature range for growth was 52-76 °C with an optimum at around 64 °C. The pH^{25C} range for growth was from 5 to 9.5 with an optimum at 7.8 - 8.4. The salinity range for growth was from 0 to 5.5 % (w/v). The utilized substrates included yeast extracts as sole carbon source and energy source, and also, in the presence of 0.1 % yeast extract, fructose and inositol. **Conclusions:** From the upper 1-9 m of sediment cores collected at the Peru Trench and Peru Margin, 10 anaerobic thermophilic isolates with slightly different properties were isolated. The strains are placed into a novel taxa, *Thermosedimentibacter oceanensis*, gen. nov., sp. nov.

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A NEW SPECIES OF ACIDOTHERMOPHILIC, OBLIGATELY CHEMOLITHOTROPHIC SULFUR-METABOLIZING ARCHAEON ISOLATED FROM AN ACIDOTHERMAL SPRING

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The archaea and bacteria mediated oxidation of sulfur to sulfuric acid is one of the major reactions of the global sulfur cycles. These microorganisms widespread in sulfur-rich environments like thermal vents, soda lakes and other solfatara areas. The order sulfolobales is a collection of extremely thermophilic, chemolithotrophic sulfur-metabolizing. Among this Sulfolobales, the genus Acidianus are characterized by facultatively anaerobic growth at high temperature and low pH in the presence of elemental sulfur and it includes three current species: A. brierleyi, A. infernus , A. ambivalens. China, especially in the southwest parts, is abundant of geothermal vents and biodiversities of these sites have not been well studied. Here we describe a new species of acidothermophilic archaeon, A. tengchongensis sp. nov. A new thermoacidophilic, obligately chemolithotrophic, facultatively aerobic archaeon Acidianus S5T, was isolated from Tengchong acidothermal spring in Southwest China. It is Gram-negative, nonmotile, irregular coccoid organism with a cell diameter of 1.2 mm. The optimal pH and temperature for growth are 2.5 and 70 oC, respectively. Under anaerobic conditions, the organism reduces elemental sulfur with molecular hydrogen, producing hydrogen sulfide. Under aerobic conditions, it oxidizes elemental sulfur and produces sulfuric acid. No growth occurs when cultivated in “iron medium”, indicating that ferrous iron cannot serve as energy source. The G+C content is 38% (mol/mol), which is much different from that of other Acidianus species (31%-32.7%). The phylogenetical distances, based on 16S rDNA sequences, to A. brierleyi, A. infernus, and A. ambivalens were 0.2, 2.6, and 2.5%, respectively. DNA-DNA hybridization rates of strain S5T to A. brierleyi, A. infernus, and A. ambivalens are 44, 22, and 23%, respectively. Thus, a new name, Acidianus tengchongensis sp. nov., is proposed for this strain S5T.

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MICROBIAL DIVERSITY OF COLD-SEEP SEDIMENTS AND MICROBIAL MATS IN SAGAMI BAY, JAPAN

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Microbial diversity of cold-seep sediments and microbial mats from Sagami Bay was determined based on 16S rDNA sequences, lipid biomarkers, and stable carbon isotopic composition of lipids isolated from the sediments. 16S rDNA sequences from the sediments and microbial mats fell into the á-, ã-, ä-, å- subdivisions of Proteobacteria, Cytophaga, Spirochaeta, and gram-positive bacteria. á-, ã-, and ä-Proteobacteria-related sequences were abundant in the *Calyptogena* sediment; whereas the ã-Proteobacteria-related sequences were abundant in the red bacteria mat sediment. The white bacteria mat sediment contained all but the å-subdivisions of Proteobacteria. Two groups of sulfate-reducing bacteria (SRB) in the ä-Proteobacteria group were identified in the *Calyptogena* sediment and the white bacteria mat sediment, but not in the red bacteria mat sediment. The archaeal 16S rDNA sequences clustered in both Crenarchaeota and Euryarchaeota. Phospholipid fatty acid and ether-linked lipid profiles of the sediments suggest the presence of SRB and archaea involved in anaerobic oxidation of methane.

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INVESTIGATING THE PIONEER MICROBIAL COLONIZERS OF HIGH TEMPERATURE PROTOCHIMNEYS IN DEEP SEA HYDROTHERMAL VENTS

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Active deep-sea hydrothermal vents are areas of intense mixing and severe thermal and chemical gradients, an extreme biotope that has been a rich resource for novel hyperthermophilic microorganisms. During expeditions in 2002 and 2003 to the hydrothermal vents of the East Pacific Rise, we used the Deep Submergence Vehicle *Alvin* to deploy titanium sampling units that allowed the growth and collection of newly formed, natural hydrothermal vents within hours of their formation. Our goal was to identify the earliest microbial colonizers of this high temperature biotope, organisms that may be lost to our view as they are replaced by a more stable climax community. Our preliminary molecular genetic data has indicated that at high temperatures, early microbial colonization is

dominated by members of the newly described Archaeal genus *Ignicoccus* and its reported parasitic symbiont, *Nanoarchaeum*. We have identified 20 unique sequences closely related to the Nanoarchaeotal group, and 5 sequences grouping closely with *Ignicoccus* from newly formed vent structures. These organisms have been found to colonize natural, high temperature protochimneys within 96 hours of their formation as well as mineral assemblages deployed over high temperature outflow. When compared phylogenetically, these colonizing organisms form a unique clade independent of those found in mature chimneys and lower temperature mineral samples. In view of the potential role of the deep sea hydrothermal vent environment in the early evolution of life on this planet, the identification of these pioneers may advance the study of how early microbial life forms gained a foothold in hydrothermal systems on early Earth and other planetary bodies.

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ISOLATION AND CHARACTERIZATION OF ANAEROBIC THERMOPHILES FROM THE UZON CALDERA

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An international, interdisciplinary Microbial Observatory Project to elucidate the microbial diversity and the dynamic relationship between thermophilic microorganisms and the biogeochemistry of the relatively unstudied of hot springs in the Uzon Caldera (Russia) is currently underway. This research will lead to a greater understanding of the biodiversity that exists in our world and the importance of microorganisms in geochemical and mineralogical processes. Part of this research includes characterizing novel glycolytic/saccharolytic anaerobic thermophiles from three different sample locations from the 'Rubber Mat Site,' (the 'Rubber Mat Site' had a temperature range from 60-90ºC and a pH value of 5.6). Enrichments were made at three different pH values (5.0, 6.0, 7.8), and were incubated at three different temperatures (60ºC, 73ºC, 90ºC), and were inoculated with hexoses, pentoses and casein as carbon/energy sources. From the enrichments at pH 7.8, fourteen rod-shaped isolates were obtained and partially characterized. Thirteen of the isolates showed terminal spore formation while only one showed central spore formation; nine of the isolates have spores that swell the cells while the other five did not. Sequencing of the 16s rRNA (using 27f and 1492r universal primers) revealed that four isolates exhibited phylogenetic distance of less than 2% to the previously described '*Clostridium uzonii*.' However, initial sequencing suggests that one of the isolates represents a novel species in the vicinity of *Thermoanaerobacter tengcongensis,* and three isolates represent novel species in the vicinity of '*Clostridium uzonii*.'

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DIVERSITY OF ULTRA-SMALL MICROORGANISMS IN GREENLAND GLACIER ICE

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Glacier ice sheets are unique extreme environments providing useful models for microbial life preserved chronologically for thousands of years and serving as analogues of possible extraterrestrial life. Studying microorganisms in glacier ice, one of the harshest environments on Earth, may provide insight into survival mechanisms at subzero temperatures, desiccation, pressure, low oxygen and nutrient concentrations. Our studies found an abundant and diverse microbial population in a sample from the Greenland Ice Sheet Project (GISP 2) taken from 3,043 m below the surface. Many cells were smaller than 1 um and could belong to the group of ultramicrobacteria (< 0.3 um in diameter). Ultra-small, free-living, microorganisms have been observed by others, but knowledge of their origin, diversity and ecological roles is limited, mostly due to difficulties in cultivating isolates. These ultra-small cells may represent not-yet-cultured intrinsically dwarf organisms or starved minute forms of known normal sized microbes. In order to add information about these cells, we examined their abundance and diversity in this Greenland ice sample. We enriched for ultra-small psychrophiles from the melted ice by successively filtering through different pore size filters, inoculating low-nutrient liquid media, and incubating in aerobic and anaerobic conditions at 5ºC. Microscopic examinations (SEM) showed an enrichment for small cells in melted ice that had passed through 0.45, 0.2, and 0.1 um filters. Flow cytometry analysis was used to estimate the number and relative cell sizes in filtered and non-filtered ice samples and in different enrichment cultures. Cloning and sequence analyses of PCR amplified Bacterial 16S rRNA genes from DNA extracted from filtered enrichment cultures showed a phylogenetic diversity with some sequences related to other dwarf or uncultured bacteria. In addition, dynamic population changes were detected during successive rounds of filtration-cultivation by community PCR profiling of the Bacterial and Archael 16S-23S IGS regions. We identified sequences related to mesophilic *Crenarchaeota* in the ice and in some enrichment cultures. Our cultivation studies indicated that the recovery of stressed and possibly damaged cells required incubations as long as 6 months. The phylogenetic representation of isolates from ice filtrates was dominated by low and high G+C Gram positive organisms, whereas predominantly *Proteobacteria* species were recovered from enrichment cultures. Possibly novel organisms were found in all major phylogenetic groups. Most isolates maintained their small cells upon recultivation and belonged to species noted for their use of diverse substrates and resistance to stresses. Our results showed that a stable population of viable, ultra-small celled organisms exists in the 120,000 year old Greenland glacier ice.

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FUNGAL DIVERSITY PRESENT AT THE SOLAR SALTERNS OF CABO ROJO, PUERTO RICO DETERMINED BY INTERNAL TRANSCRIBED SPACERS (ITS) REGIONS OF RRNA GENES CLONE LIBRARIES

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Extreme environments contain physical and/or chemical conditions that are hostile for most life forms. As a consequence of these properties, these habitats tend to have a very low biodiversity compared to more mesophilic environments. Solar salterns are one of those typical environments where sea water, trapped in pans, is evaporated through sun irradiation to obtain salt for commercial purposes. The prokaryotic diversity of both cultured and uncultured populations on these places had been extensively studied world wide. The use of culture-independent molecular analyses to study bacterial and archaeal assemblages in solar salterns have proven to be very sensitive and demonstrated that total prokaryotic diversity present at these habitats is much greater that the one obtained by culturing techniques. There are few reports that focus their attention on the fungal diversity present on these hypersaline waters. Most of these studies used typical culturing techniques as the main strategy to assess such diversity. To our knowledge, reports determining the saltern's fungal populations by the utilization of molecular microbial ecology techniques are scarce. The main objective of this research project is to assess the fungal population present in the salterns of Cabo Rojo, Puerto Rico by the use of environmental genomics. This information is valuable in order to establish comparisons with fungal strains already isolated and characterized from this environment. To determine the occurrence of fungi in these solar salterns, water samples were filtered through nitrocellulose membranes and total genomic DNA was extracted. The environmental DNA was used as template to amplify a fragment by PCR containing the 5.8S rDNA and ITS1-ITS2 regions. The resulting heterogeneous PCR amplicons were used as inserts to create an environmental ITS clone library. A total of 600 clones were obtained and, 174 were preliminary selected for further analysis. The nature of the insert present on these clones was checked by colony PCR using fungal specific ITS primers. Molecular analysis using RFLP patterns of these amplicons demonstrated that the amplified operational taxonomic units belonged to several groups. The determination of the phylogenetic relationship among these operational taxonomic units, the characterization of more clones, and the construction of a 28S rDNA clone library are currently under way. This work constitutes one of the first approaches to understand fungal diversity on extreme environments in the Caribbean.

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NITROGEN CHANGES AND BACTERIAL DIVERSITY IN SOILS OVERLYING THE UNDERGROUND CENTRALIA, PENNSYLVANIA COAL MINE FIRE

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In this study, changes to soil chemistry and domain *Bacteria* diversity were analyzed in a near-surface environment recently impacted by the Centralia, Pennsylvania anthracite coalmine fire. As this underground fire expands into new areas, land collapses are common as hot gases vent to the surface, causing rapid changes in surface soil temperatures and chemistry. In order to determine how these environmental changes are affecting the resident microbial populations, surface soil samples (at a depth of 0-20 cm) were taken from boreholes at eight locations that spanned both newly affected and unaffected areas. Soil temperature, pH, and chemical composition were analyzed at each borehole. Terminal restriction fragment length polymorphism (T-RFLP) analysis of domain *Bacteria* 16S rRNA genes and PCR with primers specific for ammonia-oxidizing bacteria were utilized to monitor the associated changes in soil microbial diversity. Over a one-year period the maximum surface soil temperatures in this site increased from 47.0°C to 75.7°C. High soil temperatures were generally associated with elevated ammonium and nitrate levels, with increased soil moisture and with decreased microbial diversity. Evidence from these experiments also suggests the presence of thermophilic or thermotolerant nitrifying bacteria in this sample site.

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DESCRIPTION OF *BACILLUS SAFENSIS*, SP. NOV. A NOVEL SPORE-FORMING BACTERIUM THAT PERSISTS IN THE SPACECRAFT AND ASSOCIATED ENVIRONMENTS

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While monitoring the microbial diversity of spacecraft associated environments over a period of 5 years (1999 to 2004), *Bacillus pumilus* was found to be the second most predominant species among aerobic spore-forming bacteria. Since *B. pumilus* showed resistance to vapor H_2O_2, a detailed taxonomic characterization of this group of bacteria was performed. Phenotypic analyses, fatty acid profiles, MALDI-TOF profiles, DNA fingerprinting, and sequencing of both the16S rRNA and *gyrB* genes divided this "problematic" species into two distinct groupings. Phenotypic characteristics and 16S rRNA gene sequence congruently placed these taxa within the genus *Bacillus,* however, such techniques could not discriminate to the species level. The closest species, *B. pumilus*, maintained a sequence similarity of >99% based on 16S rDNA sequences. However, *gyrB* sequence analysis revealed two distinct *Bacillus* species. One group of strains differed from the *B. pumilus* type strain ATCC 7061 exhibiting >10% *gyrB* sequence variation,

while the difference among strains belonging to the same group was <5%. The DNA-DNA hybridization study, a benchmark of delineating microbial species, showed only ~50% DNA relatedness between strains of these two groups while exhibiting >90% among the strains of the same species. The MALDI-TOF profile and DNA fingerprinting based on "bacterial bar coding" supported the assertion that these two groups are different and the group of strains that showed only ~50% DNA-DNA reassociation value with the *B. pumilus* type strain deserves new species status. We propose the naming of this novel *Bacillus* species, *B. safensis*, and designate FO-036b as the type strain. A PCR primer set for detection of *B. pumilus* and *B. safensis* was designed based on variations in *gyrB* sequences and ~300 strains isolated from several spacecraft surfaces (Mars Odyssey, Mars Exploration Rovers, and International Space Station) and spacecraft assembly facilities (Jet Propulsion Laboratory, Manassas, Va., Johnson Space Center and Kennedy Space Center) were screened. Approximately 18% of the strains yielded the *B. pumilus and B. safensis* -specific amplicon and DNA fingerprinting further confirmed that *B. safensis* was dominant (10%) in these spacecraft associated environments. Although *B. safensis* was isolated from both unclassified (entrance floors, ante-room, and airlock) and classified (floors, cabinet tops, and air) locations of the assembly facility, it is most predominant on surfaces in classified clean-rooms, as well as of the surfaces of spacecraft themselves.

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DIVERSITY OF BACILLI FROM HYDROTHERMAL VENTS OF EOLIAN ISLANDS (ITALY)

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Relationships between microbial communities and natural fluids in extreme marine ecosystems were investigated in shallow marine vents off the coasts of Eolian Islands (Italy). These sites, ranging in temperature from ~40 to 95°C, are rich of hot fluids emerging through fractures in the rocks of the bottom. The most active marine venting is observed off the eastern coast of Panarea Island, where gases and thermal waters reach the sea floor at depths up to 150 m over an area covering ~4 km^2. Several aerobic and anaerobic, thermophilic, microorganisms belonging to the Eubacteria and Archaea dominia were isolated from thermal springs of the Eolian Islands. Thermophilic bacilli were tested for a broad spectrum of phenotypic traits and compared with 8 thermophilic *Bacillus* and *Geobacillus* reference strains. Most of the isolates (83%) were grouped into several well-distinguished phenotipic clusters, slightly related to *G. thermodenitrificans*; remaining isolates were related to different reference strains. After the examination of DNA-DNA similarity and 16S rDNA gene sequences of 14 selected isolates, representative of different phenotipic clusters, most of them were referred to the thermophilic strains of *Geobacillus* rRNA group 5. DNA mol% G+C content determination and DNA/DNA reassociation studies allowed the identification of 5 strains as *G. thermodenitrificans*, two strains as *G. thermoleovorans* and one strain as *B. pallidus*. Hybridization studies demonstrated the presence of 6 new species as shown by the phylogenetic tree. One of the two new *Bacillus* spp. was named *B. aeolius* DSM 15084^T, the other strain was closely related to *Bacillus infernus*. Among the four new *Geobacillus* species, one was described as *G. vulcani* DSM 13174^T closely related to members of the new *Geobacillus* group 5. The remaining isolates were three novel species moderately related *G. thermoglucosidasius* and to other previously described geobacilli. Results have also shown a different distribution of species within the Eolian hydrothermal vent system. *G. thermodenitrificans* was the most dominant species among thermophilic bacilli. The new *Geobacillus* spp. and the new *B. aeolius* DSM 15084^T were ubiquitous within the Eolian system. The new species *G. vulcani* DSM 13174^T appeared to be not ubiquitous as well as its representative strains were only isolated from sediments of La Roya at Vulcano Island.

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MICROBIOLOGY OF HIGH-LEVEL NUCLEAR WASTE: CHARACTERIZATION OF THE RADIORESISTANT *KINEOCOCCUS RADIOTOLERANS*

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High-level nuclear waste (HLW) is an extreme environment consisting of a highly alkaline cocktail of concentrated alkali cations, organics, and metals, including actinides and fission products. *Kineococcus radiotolerans* was originally isolated within a shielded cell work area containing high-level radioactive waste stored in high-level caves at the Savannah River Site (Aiken, SC). This is an obligate aerobe, high G+C Gram positive actinomycete belonging to the *Kineosporiaceae* family. *K. radiotolerans* possess remarkable resistance to acute and chronic exposure to ionizing radiation, as well as prolonged desiccation. Cell survival following these pronounced insults suggests that *K. radiotolerans* is equipped with high fidelity, efficient cellular and DNA repair functions. Recent investigations have shown that *K. radiotolerans* is capable of growth on a number of meaningful organic substrates and growth is insensitive to or improved by high concentrations of inorganic anions and some metals. The draft genome sequence of *K. radiotolerans* has now been released by DOE JGI and annotated gene functions are suggestive of novel adaptations to life in a radioactive mixed waste environment. Genome sequencing and annotation have identified conventional *recA* dependent repair functions for *K. radiotolerans,* though the SOS mutagenesis pathway for translesion repair may be absent. Available genome sequence for other radioresistant microorganisms, *Deinococcus radiodurans* and *Rubrobacter xylanophilus,* presents exciting opportunities for functional and comparative genomics explorations into survival strategies in high radiation environments as well as elucidation of the evolutionary origins of radioresistance mechanisms and highly efficient DNA repair machinery. *K. radiotolerans* may offer new strategies for *in situ* bioremediation of radioactive, mixed waste contaminated environments.

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A MULTIPHASIC APPROACH TO INVESTIGATE THE MICROBIAL ECOLOGY OF A BLUE HOLE IN THE INDIAN OCEAN

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Submarine caves, cavities and niches characterized by H2S-containing vents are to be considered as extreme ecosystems that are particularly interesting for their inhabiting microflora as well as for the overall chemical, geological and biological traits. The latter are largely represented by lower eukaryotic forms that are physically distributed following the in situ concentration and gradient of micronutrients, O2 and H2S, and also the values of temperature and pH. The biota is primarily characterized by prokaryotes (both autotrophic and heterotrophic) adapted to anoxic condition and capable to form extensive biofilms on the rocky surfaces. The absence of solar irradiation, the chemo-physical traits and the fact that prokaryotes are the only living forms in specific areas of these cavities well justifies the definition of such habitats as extreme. In this work are presented the results obtained by a multiphasic approach on water, sediment and biological samples collected from a Blue Hole located in the Indian Ocean; it is characterized by a large and H2S-enriched area, by a defined chemocline at about - 40 m depth, and by a maximum depth of - 85 m. The aims of the present study were to investigate the microbial ecology of the site and to speculate on the origin of the H2S flow, yet a geological puzzle. Samples were used to inoculate enrichment media in order to investigate the most representative morphologies. Further and extensive studies were carried out by SEM and TEM analyses to determine the structural features of bacteria. The physiological patterns of microbial populations along the water column and in the bottom sediments were described by the BIOLOG system. Moreover the molecular biodiversity of the ecosystem was investigated in order to describe the distribution of microorganisms and their putative physiological roles. Preliminary data showed that sediments are particularly rich of microorganisms, mainly fermentative and sulphate-reducing bacteria. Physiological patterns showed a large metabolic response, especially in the sediments and around the chemocline, whereas less activity was detected at different layers of the water column

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MICROBIAL ENERGETICS IN YELLOWSTONE HOT SPRINGS, OR, WHAT'S ON THE MENU?

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Hyperthermophilic microbial communities in hot springs must be supported by chemical sources of energy. If the most abundant sources of energy in a given hot spring were known, this information could facilitate the culture of ecologically-relevant thermophiles and interpretation of phylogenetic surveys. To examine the energy available for autotrophic thermophiles in Yellowstone National Park, samples of more than 50 hot springs were taken for analysis of dissolved ions and gases, including 20 redox-sensitive species. The energetics of 182 inorganic redox reactions were evaluated using the calculated activities of the redox species and the standard Gibbs energies calculated at elevated temperature. Each reaction represents a potential overall metabolic reaction for a single microorganism or a consortium of microbes. Of all potential geochemical and physical differences between hot springs, pH was the strongest controlling variable in the relative energetic order of these redox reactions. At Obsidian Pool, a near-neutral hot spring where a number of microbiological and phylogenetic studies have been conducted, the activities of the oxidants (oxygen, nitrate, sulfate, etc.) controlled the relative energetics of the reactions. A comparison of the potential metabolic reactions at Obsidian Pool with the known metabolisms of cultured organisms or predicted metabolisms from phylogenetic studies revealed that some highly energetic reactions correspond to unknown metabolisms. These potential metabolisms could form the basis for culture of novel thermophiles.

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STUDIES ON THE STRESS PHYSIOLOGY OF RADIATION RESISTANT BACTERIA FROM VARIOUS HABITATS

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Radiation resistant bacteria like *Deinococcus* have polyextremophile characteristic that is they are not only resistant to radiation but also resistant to various other stresses like desiccation. The study was initiated by isolating radiation resistant bacteria from varied habitats some of which are known and some other unknown . The radiation resistance of the isolates was characterized in terms of there D10 values to gamma and U.V radiation .Their desiccation and salt tolerance was studied as well. These isolates have been further characterized with respect to the 16SrDNA sequence . RFLP analysis was carried out to verify there relatedness amongst each other.16SrDNA sequence analysis indicate great diversity amongst these isolates. Catalase and superoxide dismutase are oxidative enzymes known to play a role in the radiation physiology of the organism. These enzymes play an important role in other

stresses.Analysis of these enzymes under gamma, desiccation and salt stress revealed a two to three fold increase in the activities, confirming their role in these stress physiology. Protein profile of these isolates pre and post stresses will be presented.

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BIOCHEMICAL INDICATORS AND ENZYMATIC ACTIVITY BELOW PERMAFROST ENVIRONMENT

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Permafrost- perennially frozen ground represents a unique stable, physical-chemical environment that has allowed the prolonged survival of microbial lineage at subzero temperatures incomparably longer than any other known habitats. Recently, the interest in the limit of life in low temperature environments has been growing. It is known that permafrost contains a large number of a variety of ancient viable microorganisms (Vorobyova et al., 1997). Viable microorganisms (aerobes and anaerobes, cyanobacteria and green alga, yeast and fungi) were isolated from sediments, which were maintained in a frozen state for extended periods. It is the only microbial community known to have retained viability over geological times and, upon thawing, to be able to renew their physiological activity. The mostly psychrotolerant community, has been described as "a community of survivors" (Friedmann, 1994), with the starvation-survival lifestyle as the normal physiological state (Morita, 2000). Microbial activities and biochemical indicators are correlatively interacted each other, hence systematical study of permafrost environment are required in terms of extremophilic biogeochemistry. Here we present that core samples of terrestrial sediments at depths of 0 - 300 cm at Rikubetsu, Hokkaido, Japan were analyzed for the determination of product moment correlation coefficient (r) regarding amino acids, amino sugars, total organic carbon, total sulfur, enzymatic activities and microbial cell density. Abundance of sedimentary organic matters and the density of viable microorganisms were greatest at the surface and drastically decreased with the depth. However, D/L ratio of chiral amino acids and non-proteinous amino acids such as beta-alanine and gamma-aminobutyric acid showed negative correlation with the depth. Hence, racemization reactions and the alteration of dicarboxylic amino acids, aspartic acid and glutamic acid, to beta-alanine and gamma-aminobutyric acid respectively, via specific decarboxylation due to diagenesis were observed over the past 10,000 years. Vertical distributions of biomarkers are highly consistent with the subterranean microbial activities in the sediment.

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MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF FREE-FLOATING FILAMENTOUS CYANOBACTERIAL MATS FROM GEOTHERMAL SPRINGS IN THE PHILIPPINES

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A novel cyanobacterial mat type is characterized from near-neutral pH, low sulphide geothermal springs of 45-60°C in the Philippines. Mats were free floating, several metres in diameter and several cm in thickness. The upper surface of mats was covered in a waxy scytonemin-like layer, solvent extracts of which absorbed light strongly at 384nm. Light microscopy revealed mats to posses highly ordered layers of air spaces at both the macroscopic and microscopic level, apparently as an adaptation to buoyancy. Morphospecies composition was exclusively filamentous, with *Fischerella*-like and *Oscillatoria*-like taxa closely associated throughout mats. Abundant heterocystous cells were observed in *Fischerella* filaments, suggesting nitrogen fixation occurs in these mats. Morphological structure did not vary among mats from pools of different temperature, but several 16S rDNA-defined genotypes were resolved by DGGE with some displaying greater thermophily than others. Sequencing of fourteen DGGE bands (Genbank accession numbers: AY236467-AY236480) yielded nine novel *Fischerella* sequences, whilst the five *Oscillatoria* sequences showed high similarity to other thermophilic *Oscillatoria* sequences. These data are relevant to astrobiology in that they expand our knowledge of oxygenic photosynthetic community diversity in geothermal environments, which serve as modern analogues for early life on Earth and other planets.

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DIVERSITY AND COLD ADAPTATION OF *PEDOBACTER* SPP. ISOLATED FROM FINNISH LAPLAND AND SVALBARD

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Pedobacter spp. are members of the *Cytophaga/Flexibacter/Bacteroides* group and have been isolated from various soil and alpine glacier environments. We isolated and characterized 31 *Pedobacter* strains from soil and lichen samples collected from six sites in Finnish Lapland and Svalbard. The isolates were most closely related to *Pedobacter cryoconitis* (21 strains), *P. heparinus* (5 strains) and *P. africanus* (1 strain) as judged by their REP-PCR patterns and 16S rRNA gene sequences. Three of the strains were less than 92% similar to any known species. All of the *Pedobacter* isolates grew at 0°C within 2 weeks of incubation and possessed various hydrolytic enzyme activities at 5°C, including cellulase, xylanase, lichenase, amylase and/or protease activities. Moreover, the majority of the strains (n=21) produced copious amounts of extracellular polysaccharides

(EPS) which may have a role in their cold tolerance. One of the EPS producing strains, ANJC1, was selected for further characterization. This strain shared 98% 16S rRNA gene sequence homology with *Pedobacter cryoconitis*. Growth rate, EPS production and fatty acid composition of ANJC1 were monitored at different temperatures. Strain ANJC1 grew well down to -5°C with a doubling time of approximately 4 days at this temperature. Exopolysaccharide production at 0, 10 and 25°C ranged from 1.5 to 2.3 mg EPS/mg of biomass and was slightly higher at the lower temperatures. Cold induced membrane fatty acid adaptation in this strain included unsaturation of C_{16} fatty acids, increase of iso- and a slight decrease of anteiso-branched fatty acids. *Pedobacter* strains were isolated from nearly all soil samples collected from Northern Finland and Svalbard. This together with their ability to degrade various plant polysaccharides and good growth at sub-zero temperatures indicate that they are common and well adapted to the cold and humic Northern soils.

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PHYLOGENETIC AND BIOCHEMICAL CHARACTERIZATION OF TWO MICROBIAL ISOLATES FROM THE LICANCABUR VOLCANO CRATER LAKE

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We are currently investigating the biological population present in the highest and least explored perennial lakes on earth, located in the Bolivian and Chilean Andes, including several volcanic crater lakes of more than 6000 m elevation. Our samples were collected in saline lakes of the Laguna Blanca - Laguna Verde, areas located in the Bolivian Altiplano and in the Licancabur volcano crater (27°47'S/67°47'W). In the ongoing project we attempt to study high altitude lakes. The main goal of the project is to look for analogies with Martian paleolakes. These Bolivian lakes under study can be described as Andean lakes following the classification of Chong. From several samples taken during the 2003 Licancabur volcano expedition, we have attempted to isolate pure cultures using a combination of microbiological and molecular biological methods. Here we present the current results of the microbiological, biochemical and phylogenetical characterization of two prokaryotes we have grown in the laboratory.

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EVOLUTION OF THE ARCHAEA-DERIVED PUTATIVE OLIGOPEPTIDE ABC TRANSPORTERS IN THERMOTOGA MARITIMA

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The *Thermotoga maritima* chromosome reportedly encodes eleven putative oligopeptide ABC transporters arranged in apparent operons each encoding three components: a substrate binding protein (SBP), two membrane-spanning proteins (MSP), and two ATP-binding proteins (ABP). Simple BLAST analyses of translated products of these genes suggested that six of these putative operons are most closely related to archaeal homologs. We have performed detailed phylogenetic analyses of these protein sequences to determine their evolutionary histories. For our dataset, we chose those bacterial and archaeal homologs whose genes are adjacent in their respective genomes. Alignments of the three components showed that unlike the MSPs and ABPs, only closely related SBPs could be aligned with confidence demonstrating the SBP sequences evolve faster than those of the other two components. Parsimony analysis of the four individual proteins' datasets (two MSPs and two ABPs) and a dataset of concatenated sequences of all four proteins showed that the trees derived from the individual proteins were not all congruent with the concatenated dataset tree. Distance matrix analyses using maximum likelihood distances of the four individual protein datasets and two datasets representing concatenations of the two MSPs and two ABPs showed that while the MSP concatenated tree reflected the same evolutionary pattern of its individual proteins, the ABP concatenated tree did not. Consequently it appears that the ABPs have an evolutionary history significantly different from that of the remaining *T. maritima* sequences in the dataset. Trees derived from parsimony and maximum likelihood distance matrix methods demonstrate that these six operons are derived from the Archaea and arrived in the *Thermotoga* lineage *via* at least two horizontal transfers, one from the pyrococci and the other from either *Archaeoglobus* or *Aeropyrum*. There is evidence from other *Thermotogales* species that some of these operons have been subsequently transferred vertically through the lineage. There is also evidence that intraoperon recombination has occurred. In particular one operon is interrupted in *T. maritima* by a â-galactosidase gene derived from the *Thermus* lineage. Evidence that these transporters have changed to operate within a bacterium is provided by the fact that the SBPs have lost the C-terminal hydrophobic domain found in archaeal oligopeptide SBPs. This study demonstrates that this family of ABC transporter operons was derived from the Archaea and the individual operons have evolved as units at least in part through further horizontal gene transfer events.

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MICROBIAL COMMUNITIES ASSOCIATED WITH FINES FROM A NORTH SEA PETROLEUM RESERVOIR

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During oil production a considerable amount of undefined fines have been detected in the oil from a hot North Sea petroleum reservoir (temperature > 80°C), corresponding to a daily weight of 15 tons (oil production 1000 m^3 oil/day). The origin of these fines has not been clarified. One suggestion is that these fines are residues of biofilms from the near-well area detached during oil production. Initial studies showed prokaryote-like particles in the fines, although no culture could be performed. Analysis of proteins, total carbohydrates and fat indicated possible contents of biological materials in the samples. This reservoir had no previous recorded microbial history. Nucleic acids were extracted from oil, formation water, emulsion and dry filters originating from different wells (n=10), several of these with no injection water penetration. Partial bacterial or archaeal 16S rDNA sequences were amplified by PCR. No positive archaeal PCR products were recorded, while 48 of 49 samples were positive with bacterial primers. DGGE analysis revealed a few predominant bands (1 - 4). Thirteen samples, including water, emulsions, filters, and oil phases, were selected for cloning and restriction fragment length polymorphism (RFLP) analysis by two restriction enzymes (*Hae*III and *Rsa*I). A library of 70 clones was generated, and these distributed within 25 RFLP types. About half of the clones (54%) distributed within a few RFLP types (n=5) containing clones from both water and oil phases. Sequence analysis and alignments of 22 clones representing the predominant RFLP types revealed that the clones distributed within proteobacteria (á-, â-, ã-, or å-subgroups) or within the thermotogales, with predominance of ã-, and å-proteobacteria. Clones originating from the water phase samples showed homologies to *Arcobacter* sp. and *Sulfospirillum arcachonensis* (å-proteobacteria), *Halomonas* sp. (ã-proteobacteria), and *Geotoga petrea* (thermotogales). All these microbial groups have been reported from oil reservoirs in other parts of the world. In conclusion, our findings indicated that the fines produced in large amounts from a hot offshore reservoir may be associated with indigenous microbial growth.

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CHARACTERIZATION OF FILAMENTOUS FUNGI ISOLATED FROM EXTREME ENVIRONMENTS

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Over a period of almost two decades, large numbers of filamentous fungi were isolated from extreme environments in the USA and the territory of the former Soviet Union, including but not limited to deserts and former military bases in California, the failed nuclear power plant and the surrounding exclusion zone in Chernobyl, Ukraine, Lake Baikal and the Kamchatka peninsula in Russia. These environmental field sites were considered extreme in terms of their water activity, xenobiotic contamination, temperature, geology or chemistry. Published media and isolation techniques were modified and applied to emphasize the importance of simulating the eco-physiological conditions at the environmental niche in succeeding with fungal isolation. Pure cultures of filamentous fungi were preserved and stored at ultra-low temperature for long-term strain maintenance. Characterization of fungal organisms was carried out using traditional, colony- and cell morphology-based taxonomical methods, as well as molecular-level protocols. A comparative analysis of fatty acid methyl ester (FAME) profiling, genomic DNA sequencing of specific target genes valuable for phylogenetic differentiation and inter- and intraspecies comparison, and classical identification will be presented. Furthermore, the ecological significance of filamentous fungi in extreme environments, the state-of-the-art of fungal classification methods, and the potential of these fungi for biotechnology and biomedical research will be discussed.

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CHARACTERIZATION OF MICROBIAL COMMUNITIES IN PETROLEUM RESERVOIRS BY CULTURE-DEPENDENT- AND CULTURE-INDEPENDENT METHODS

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The microbial diversity of two North Sea reservoirs has been studied in some detail by culture-dependent and culture-independent methods, and both a culture collection (the Reservoir Strain Collection - RSC) and a 16S-rDNA library have been established for these reservoirs. Samples have been collected from both produced water, exploration cores, fines produced with the oil, and in separation tanks. Success in isolating and cultivating has only revealed a small fraction of microorganisms shown in reservoir samples by culture-independent molecular biology methods. In particular samples from one North Sea oil field have been difficult

to propagate in culture. The growth media employed were commonly used synthetic media, generally quite rich in ready carbon sources, and may be devoid of essential compounds that are present *in situ*. Novel culturing methods for reservoir organisms are being implemented, which aim to simulate the reservoir conditions as far as possible regarding media composition, oil and geochemistry, temperature and pressure. The modified media are based on synthetic brine (specific for the actual field) supplied with appropriate nutrients. In addition, oil and, if possible, sediments from the actual field will be employed. Growth temperature will be the actual field temperature. Pressure-chambers for cultivation have been constructed, and pressurized fluid (water) has been transferred to these new pressure-chambers at 250 bars. Protocols for cultivation under pressure will be tested out using organisms from the RSC. Partial bacterial and archaeal 16S rDNA sequences from different reservoir samples were amplified by PCR, and analyzed by DGGE and cloning-sequencing strategies. Preliminary results indicate the presence of a variety of organisms in the samples, and significant differences between reservoirs. Within each reservoir some of the microbes found may be regarded as "key" organisms. These may be important for the explanation of biological activities in their reservoirs. In order to balance the beneficial and detrimental effects of microbial activity in the reservoir, new knowledge is required.

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PHYLOGENETIC ANALYSIS OF MICROBIAL DIVERSITY IN ANARCTIC COLD-DESERT BIOTOPES

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Some of the most extreme environments thought to harbour life are in the Dry Valleys of South Victoria Land, Eastern Antarctica. This region is subject to extremely low seasonal temperatures (-30 - 50°C), wide temperature fluctuation, high winds, desiccation, and high incident radiation. The Dry Valleys largely composed of oligotrophic gravels, the upper profiles of which have an average water content of 0.5 - 2% wt. Soils from two Dry Valley regions, one an exposed low altitude sample and the other a high altitude sample collected near a frozen lake margin, were sampled aseptically. A third sample of ornithogenic origin representing both a nutrient- and water-rich environment was taken from near Bratina Island. All three samples were subjected to molecular phylogenetic analysis in order to assess prokaryote diversity. Prior assessments conducted in our laboratory on microbial abundance in Arctic soils were based on ATP analysis and indicated prokaryotic cell numbers to be between 10^6 - 10^8.g^{-1}. DNA extractions were performed on all three soil samples and 16S rDNA clone libraries were constructed using universal 16S rDNA primers E9F and U1510R. Over 200 colonies were subjected to ARDRA analysis and were selected for sequencing. The results obtained from partial BLASTN analysis indicated 9 defined phylotypes. Distribution varied as certain phylotypes such as cyanobacteria appeared unique and other phylotypes, such as Actinobacteria, seem cosmopolitan. Of the three soils cyanobacterial signals were limited to the high altitude desiccated mineral soil, which is uncharacteristic of cyanobacteria for they are associated with moist soils and aquatic biotopes. This suggests that determining soil water content as % wt only indicate water levels available at a given time, but is a poor measure of free available water. Actinobacteria, known for their role in humus formation, were evenly prevalent throughout all three soils even though their presence in dry mineral soils would not be expected. Acidobacteria appeared to be restricted to the low altitude desiccated and nutrient rich soils, as no acidobacterial signals were detected in the high altitude soil. A large portion of acidobacteria are still unknown as they form a major part of non-cultured bacteria in most soils studied. No distinct bacteria were identified in the nutrient rich soil except that it proportionally contains the largest number of uncultured phylotypes. The remainder of clones (43%) show distant to close identity to uncultured, unidentified or taxonomically unclear phylotypes. A total of six phylotypes were identified down to species level, two were cyanobacteria and three actinobacteria one of which is a type strain. No contamination signals such as *Staphylococcus epidermis* or *E. coli* was detected.

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PIEZOPHILES IN JAPAN SEA AND KURIL TRENCH Â€"THE BIOGEOGRAPHY OF PIEZOPHILES-

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Piezophilic bacteria, which adapt to high hydrostatic pressure, were isolated from the deep-sea sediments at the limited number of diving sites. To study the piezophilic bacterial diversity, we organized the cruise during 2002 to 2003, for obtaining the deep sediment samples from two different cold seep sites at the bottom of Japan Sea and the Kuril Trench. Japan Sea is believed as a separated ocean from the other ocean area, like Pacific Ocean, Eastern Chinese Sea, etc. Because of the connection sites (strait or channel) between Japan Sea and other oceans are pretty shallow, the transfer of deep-sea animals from the other ocean tend to be prevented. Therefore, the deep-sea piezophilic microbiological diversity were investigated to confirm whether deep-sea bottom of Japan Sea is separated with other oceans. At the other hands, huge chemosynthetic animal communities with clams (Calyptogena sp.), carbonate chimneys and bacterial mats were newly found on the slope of the subducted Cadet Seamount at the southern end of the Kuril Trench forearc. The diversity of piezophiles were also examined. The deep sediment samples from the bacterial mat in the cold seep site were collected from the depths of 3,100 m at Shiribeshi Trough in Japan Sea and 4,700 m at the Kuril Trench. The mix cultivation of sediment samples with high hydrostatic pressure was performed using DEEPBATH (deep-sea high-pressure microorganisms isolation and cultivation system, JAMSTEC) system, which can retain pressure during cultivation. With samples from Japan Sea, the microbial community in the deep sediment samples obtained from the bottom of Japan Sea changed during the high-pressure cultivation. Based on the results of t-RFLP analysis, only the genera Moritella, Shewanella and Psychromonas grew under such conditions (30 and 50 MPa). The phylogenetic relations of the

16S rDNA sequences from those isolates suggested that they were closely related with piezophilic bacterial groups isolated from Pacific Ocean bottoms. In addition, the growth profiles of those isolates were shown as piezophilic properties. Therefore, these results suggested that pressure-adapted microbial diversity in the deep Japan Sea could be similar to that in the deep Pacific Ocean, which is different from deep-sea animals. In case of Kuril Trench, the phylogenetic relations of the 16S rDNA sequences from the DEEPBATH mix culture suggested the possibility of piezophiles which belongs to the different genus of already known. The subducted Cadet Seamount at the Kuril Trench may be estimated to cause some difference of the fluid to sustain the piezophilic bacterial community.

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RECOMBINATION IN NATURAL POPULATIONS OF SULFOLOBUS ISLANDICUS FROM THE MUTNOVSKY VOLCANO

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We examine the natural population structure of thermoacidophilic crenarchaea Sulfolobus islandicus. While a diversity of viruses and plasmids of Sulfolobus species have been suggested to promote genetic exchange under laboratory conditions and in nature, the level of genetic exchange in wild Sulfolobus populations has never been explored. We performed multilocus sequence analysis of six variable protein-coding loci from 60 S. islandicus strains from Mutnovsky Volcano of eastern Russia. Significant incongruence among gene genealogies and a low level of association between alleles show that genetic exchange plays a vital role in generating genomic diversity in this wild population. It is estimated that any nucleotide is up to 100 times more likely to change by recombination than by mutation in the Mutnovsky S. islandicus population. The effect of recombination on the evolution and maintenance of genetic diversityin the unstable, extreme environment of geothermal hot springs is discussed. We suggest that the high level of allelic diversity found in the Mutnovsky hot springs may result from historical admixture with the S. islandicus population from the Uzon/Geyser Valley geothermal region of Kamchatka.

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NANOARCHAEOTE PHYLOTYPES DOMINATE IN CHINESE HYDROTHERMAL BIOTOPES

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The Nanoarchaeota was proposed as the fourth sub-division of the Archaea in 1992. The first, and only fully characterised, Nanoarchaeon was found existing in symbiosis with the Crenarchaeote, *Igniococcus*. This Nanoarchaeote, named *N. equitans*, did not amplify with "standard archaeal 16S PCR primers" and could only be amplified using specifically designed primers. Using these Nanoarchaeote-specific primers two further phylotypes were amplified from hydrothermal environments. We have designed a new set of "universal archaeal primers," that amplify the 16S gene of all four archaeal sub-divisions. Using these primers we have amplified community DNA from a Chinese hydrothermal system and discovered that the dominant phylotypes are Nanoarchaeal.

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CULTIVATION OF A REPRESENTATIVE OF NOVEL *CRENARCHAEOTA* FROM SAMPLES OF LAKE BAIKAL REGION

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Hot springs of Kamchatka and Lake Baikal region, Russia, were investigated for the presence of the representatives of the kingdom *Crenarchaeota* using the 16S rRNA gene as a molecular marker. Eleven samples of water and sediments from Kamchatka hot springs with the temperature from 50ºC to 84ºC and pH from 4.3 to 6.8, and seven samples of water and sediments from the hot springs of the Lake Baikal region with the temperature from 59ºC to 71ºC and pH from 8.4 to 8.9 were studied. Microbial DNA extracted directly from natural samples was subjected to PCR amplification with the Crenarchaeota-specific primers 7F-518R (1). The representatives of the kingdom *Crenarchaeota* were detected in seven Kamchatka samples and in a one sample from the Lake Baikal region with the temperature 59.1ºC and pH 8.8 (sample 1017). The sequence of PCR product from the sample 1017 showed the presence of one representative of a deep-branching uncultivated crenarchaeota group, earlier detected in Yellowstone National Park and represented by the environmental clone pJP41 (2). The enrichment cultures obtained on different substrates, at various temperatures and pH from sample 1017 were monitored by PCR with the Crenarchaeota-specific primers 7F-518R. It allowed us to describe the optimal growth conditions for the representative of crenarchaeota present in sample 1017. It appeared to be the strict anaerobe, organotroph independent on the presence of electron acceptors, with the temperature range of growth from 60ºC to 65ºC and pH range of growth from 7.0 to 7.5. Thus, using the Crenarchaeota-specific primers and the cultivation approach, we managed to give the primary phenotypic characteristic of the representative of the deep-branching phylogenetic group of *Crenarchaeota* known before as uncultivated organisms.

1. Perevalova A.A., Lebedinsky A.V., Bonch-Osmolovskaya E.A., Chernyh N.A. (2003). Microbiology (English translation of Mikrobiologiya), 72 (3): 340-346
2. Barns S.M., Delwiche C.F., Palmer J.D., and Pace N.R. (1996). Proc. Natl. Acad. Sci. USA. 93(17): 9188-9193

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SRB IN AN EXTREME ACIDIC SYSTEM, THE TINTO RIVER

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The Tinto River, a 100 km-long river in Southwestern Spain, is an extreme acidic ecosystem (pH between 1.5 to 3.1) and high concentration of heavy metals in solution (1). The river rises at Peña de Hierro, in the core of the Iberian Pyritic Belt and flows into the Atlantic Ocean at Huelva. It gives its name to an important mining region, which has been in operation for over five thousand years (1). The extreme conditions of the Tinto ecosystem are generated by the metabolic activity of chemolithotrophic microorganisms thriving in the rich complex sulfides of the Iberian Pyritic Belt (2). Molecular ecology techniques showed the presence of microorganisms related with the iron cycle, *Leptospirillum* spp., *Acidithiobacillus ferrooxidans* and *Acidiphilium* spp., but in some locations sulfate reducing bacteria (SRB) were detected (3). The presence of SRB in this extreme acidic system was analyzed using molecular and conventional microbiological techniques (FISH, 16S rRNA gene cloning and enrichment cultures). The Gram positive sulfate reducing bacteria *Desulfosporosinus* spp. was detected and isolated in different parts of the Tinto River. Bioremediation of acidic, metal-polluted waters using SRB has been shown in pilot scale operations even under acidic condition (4, 5), but the ecology of this kind of bacteria in acidic extreme ecosystems has not been elucidated. In this study the ecology of SRB in the Tinto ecosystem and their role in the sulfur cycle is analyzed.

1. Dvorak, D., R. Hedin, H. Edenborn, and P. Mclntyre. 1992. Treament of metal contaminated water using bacterial sulfate reduction: results from pilot-scale reactor. Biotechnol Bioeng. 40:609-616.
2. González-Toril, E., F. Gómez, N. Rodríguez, D. Fernández, J. Zuluaga, I. Marín, and R. Amils. 2003. Geomicrobiology of the Tinto river, a model of interest for biohydrometallurgy. Hydrometallurgy. 71:301-309.
3. González-Toril, E., E. Llobet-Brossa, C. E.O., R. Amann, and R. Amils. 2003. Microbial ecology of an extreme acidic environment, Tinto River. Applied and Environmental Microbiology. 69:4853-4865.
4. Kolmert, Å., and D. B. Johnson. 2001. Remediation of acidic waste waters using immobilised, acidophilic sulfate-reducing bacteria. Journal of Chemical Technology and Biotechnology. 76:836-843.
5. López-Archilla, A. I., I. Marín, and R. Amils. 2001. Microbial community composition and ecology of an acidic aquatic enviroment: the Tinto River, Spain. Microbial Ecology. 41:20-35.

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MICROBIAL ECOLOGY OF SUBMARINE CAVES EXHIBITING EXTENSIVE ANOXIC NICHES DUE TO H_2S-CONTAINING VENTS

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Submarine caves are usually described as non-conventional and shallow marine ecosystems due to the absence of sun irradiation that lead to a poor development of biota if compared to common coastal habitats. In the South of Italy, along the coast between Campania and Basilicata, the Cape of Palinuro is characterised by numerous submarine caves and few of them show inner H_2S-enriched vents. The "Grotta Azzurra" and the "Grotta Sulfurea" are the two caves exhibiting the most significant venting activity and the development of anoxic niches is remarkable. Anoxic fresh-waters are clearly separated by aerobic sea-waters throughout a definite chemocline; eukaryotic life exists only in the oxygenated water layers, whereas over the chemocline no living forms but microorganisms are observed. During the last decade several investigations were carried out on these caves as far as regard the autoctonuos microflora and preliminary data were obtained on the population structure by means of SEM analyses. Following experiments were focused on the metabolic patterns exhibited by microorganisms in different niches and on the isolation of the most representative heterotrophic bacteria. Recently further electron microscopic studies were performed and molecular DNA analyses were carried out to get new insights into the microbial composition of the overall prokaryotic population. The distribution of microbial populations were described in terms of 16S rDNA sequences and the predominance of either prokaria or archaea in different niches was investigated. The results presented in this work are with no doubt a significant achievement in order to describe the microbial ecology of an unique ecosystem; however our data may also represent an important step towards the understanding of the biology of both submarine caves and other non-conventional marine habitats.

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DIVERSITY AND ECOLOGY OF VIRUSES AND PROKARYOTES IN A SOLAR SALTERN SYSTEM

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The series of ecosystems encompassed by multi-pond solar salterns allows for the study of phage-host dynamics across very large diversity, environmental, and concentration gradients. In salt crystallizer ponds, prokaryotes reach concentrations of up to 10^8 cells per ml. Despite a significant increase in overall concentrations along the salinity gradient, there is a constant ratio of ~10 phage particles per prokaryotic cell in each pond of the saltern system. We have performed genomic and ecological analyses of the uncultured

phage, *Bacteria*, and *Archaea* throughout a multi-pond saltern system. The concentrations and production rates of phage and prokaryotes were determined for each of the saltern ponds. Metagenomic analyses of phage and prokaryotic communities from the salt crystallizer pond suggest that diversity of phage and hosts does not increase with increasing encounter rates. A combination of experimental data and mathematical modeling has shown that spatial heterogeneity is critical for modulating phage-host population ratios over changing microbe concentrations.

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DIFFERENTIAL SCANNING CALORIMETRY OF WHOLE CELLS AND RIBOSOMES OF HYPERTHERMOPHILIC ARCHAEON

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When micro-organisms are heated a reproducible sequence of endothermic or exothermic processes occurs. These perturbations can be observed by differential scanning calorimetry (DSC). The technique detects and records a characteristic pattern of peaks, corresponding to specific denaturation processes, as a time-temperature sequence *(1)*. To date there are few publications describing DSC of vegetative bacterial cells *(2-5)*. There are no reports on DSC of whole cells of hyperthermophilic organisms. In our study *Aeropyrum pernix*, the first reported obligately aerobic and neutrophilic hyperthermophilic archaeon was used. It belongs to *Desulfurococcales* order in *Crenarchaeota* kingdom. Its optimum growth temperature is between 90 and 95 °C and the optimum pH for growth around 7. It grows at a salinity from 1.8% to 7.0% with optimum at around 3.5% *(6)*. To obtain a characteristic thermogram we ran DSC on whole cells pellet. Reversibility of transitions was tested with preheating to different temperatures. We wanted to evaluate the influence of the environmental factors (pH) on the shape of the thermogram and on peak temperatures. DSC was performed after growing the organism at its optimal pH and washing the cell pellet with buffers of different pH values. These results were compared to thermograms of cell pellets after actual growing at different pH. It is known for bacteria, that the major DSC peak is the result of the danaturation of ribosomes. With the purpose to check if this was also true for *A. pernix*, we isolated its ribosomes and ribosomal subunits and ran DSC on them. DSC thermograms of untreated *A. pernix* pellets, after growing at pH 7 and washing with different buffers, show several endothermic transitions over a temperature range of 110 to 150 °C. Preheating showed partial reversibility of some transitions to 135 °C. On the characteristic thermogram, clearly there are two major peaks with peak temperatures around 120 and 130 °C. Both washing the pellet with different buffers and growing the organism at different pH affected peak temperatures and the shape of the thermogram. pH below 7 had stronger effect to the second major peak, while greater influence was observed on the first major peak at pH above 7. Results of the DSC of isolated ribosomes and ribosomal subunits imply that denaturation of ribosomes lies behind the two major peaks of characteristic *A. pernix* thermogram.

(1) Mackey , B. M. et al., 1991, Journal of General Microbiology, 137, 2361-2374.
(2) Miles, C.A. et al., 1986, Journal of General Microbiology, 132, 939-952.
(3) Teixeira, P. et al., 1997, Journal of Applied Microbiology, 83, 219-226.
(4) Mohácsi-Farkas, Cs. et al., 1999, Journal of Thermal Analysis and Calorimetry, 57, 409-414.
(5) Lee, J. and Kaletunç, G., 2002, Applied and Environmental Microbiology, 68, 5379-5386.
(6) Sako, Y. et al., 1996, international Journal of Systematic Bacteriology, 46, 1070-1077.

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DISSIMILATORY ARSENATE REDUCTION IN SEDIMENTS AND CULTURES FROM SEARLES LAKE, CALIFORNIA: AN ARSENIC-RICH, SALT-SATURATED SODA LAKE

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Searles Lake is an alkaline (pH = 9.8), salt saturated (sal. = ~ 300 g/L) soda lake located in the Mohave Desert of California. It contains extremely high concentrations of dissolved arsenic oxyanions (~ 3.0 mM) and sulfate (~ 800 mM). Porewater profiles taken from anoxic, saltern sediments indicate a complete shift from the + 5 oxidation state (arsenate) in the overlying water to the + 3 oxidation state (arsenite) with depth. Anaerobic sediment slurries demonstrated a biological removal of 1 mM arsenate that was accelerated with H_2. A pure culture of a curved rod, designated strain SLAS-1, was isolated that grew by oxidizing lactate to acetate plus CO_2 with the reduction of arsenate to arsenite. The culture grew only at high salinities (200 - 330 g/L) and high pH (≥ 9.1), but with a slow growth rate (t_d = ~ 1 day). Phylogenetic alignment made by 16S rDNA sequences indicated this strain was a member of the Haloanaerobiales, but having no closely related species (≤ 81 % sequence identity) either in culture or detected from amplification of DNA in the environment. Strain SLAS-1 exhibited an unusual wave-like motility, but had no obvious external flagella. TEM thin sections revealed the presence of a cytoplasmic, curved axial filament running the length of the organism that we hypothesize may be involved in its motility. In an effort to isolate As-respiring Archaea, we established a lactate-arsenate-sulfate enrichment in the presence of the selective pressure of several antibiotics. The enrichment, SERL-AB, consists of a lactate-oxidizing sulfate-reducer in co-culture with sulifde-oxidizing arsenate-reducer. Collectively, these results support the presence of the reductive portion of an arsenic cycle in these salt-saturated sediments. Since nitrate is also abundant (~ 1 mM) in some of the saltern waters, anaerobic oxidation of arsenite to arsenate with this oxidant is at least theoretically possible. Hence, the occurrence of a complete arsenic cycle in this ecosystem remains a subject for future investigation.

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ARCHAEAL VIRUSES FROM HIGH TEMPERATURE ACIDIC ENVIRONMENTS

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Our interest is the isolation, identification and molecular characterization of archaeal viruses from high temperature (> 80) acidic (pH<4.0) environments found in Yellowstone National Park USA and other thermal area worldwide. For one such virus, STIV, we have recently determined the sequence of its dsDNA genome and the structure of the viral particle. Detailed analysis of its genome predicts several regulatory proteins not typically found associated with viral genomes. The structure of the virus particle reveals a morphology not previous observed for any other virus. However, the structure of the major capsid protein shows relationships to other capsid proteins associated with the other two domains of life. This suggests that this viral architecture may be ancient and may have preceded the separation of life into the three recognized domains. We have also been examining how archaeal viral populations change over time in Yellowstone thermal environments. We have been monitoring changes in the SSV, SIRV, and STIV populations in three thermal features approximately every 30 days for more than a year by analysis of PCR cloned libraries made directly from environmental samples. Surprisingly, the virus populations are remarkably dynamic with major shifts occurring within a relatively short time period. In addition, changes at one monitor site are independent of changes at the other sites. We speculate that the dynamics in viral populations is driven by changes in the geochemistry and host populations unique to each thermal feature.

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TTSV1, A NOVEL VIRUS ISOLATED FROM THE HYPERTHERMOPHILIC *CRENARCHAEOTE, THERMOPROTEUS TENAX*

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The novel virus TTSV1 was isolated from the crenarchaeaotal hyperthermophile *Thermoproteus tenax* sampled at a hot spring in Indonesia. While viruses isolated previously from *T. tenax* are stiff or flexible rod type, TTSV1 has a spherical shape with a diameter of 70 nm. TTSV1 has an envelope and a density of 1.29 g/ml. Three putative viral structural proteins of 10, 20, and 35 kDa in size were identified. TTSV1 was present in a stable carrier state in its host cells, which produce viruses without noticeable cell lysis. TTSV1 contains an approximately 21 kbp linear double-stranded DNA genome, the ends of which are not linked with a protein. The viral genome was not integrated into the host chromosomal DNA and partially methylated at cytosine residues. Sequencing of approximately 85% of total viral genome revealed no significant homology in nucleic acid sequences compared with other viruses, including TTV1 isolated from *T. tenax*. TTSV1 genome has a G+C content of ~50%. Amino acid sequences of putative open reading frames deduced from the partial viral genome sequence showed little or no similarity to genes in the public databases. TTSV1 is the first spherical shape virus isolated from *T. tenax*, and should be classified in a new group along with the morphologically similar uncharacterized virus-like particles recently found in thermal environment of Yellowstone National Park, USA.

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MERCURIC REDUCTASE ENZYMES FROM MESOPHILIC BACTERIA ARE OPTIMALLY ACTIVE AT A MODERATELY THERMOPHILIC TO THERMOPHILIC TEMPERATURE RANGE

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It has been long postulated that microbe-mercury interactions originated in geothermal environments. To test this hypothesis we determined the temperature optima for the activity of the mercuric reductase (MR) enzyme. The MR is at the core of the bacterial, and possibly archeal, mercury resistance system by catalyzing the reduction of the highly toxic form of mercury (Hg(II)), to the volatile less toxic form (Hg(0)). Mercuric reductases representing the known phylogenetic diversity of this enzyme among bacteria were assayed for the effect of temperature on the specific rate of Hg(II) dependent oxidation of NADPH. Results showed optimal activities for all tested MR enzymes at the moderately thermophilic to the thermophilic range, with the enzymes from Staphylococcus aureus at 45 °C and that of transposon 21 (Tn21), which was originally isolated from Shiegella flexneri, at 65 - 70 °C. The enzymes from Bacillus cereus, Tn501 that originated in Pseudomonas aeruginosa, and Acidithiobacillus ferroxidans were optimally active at 55 - 65 °C, 55 - 60 °C, and 60 - 65 °C, respectively. As all strains in which these MR enzymes originated were mesophilic our findings show that the temperature profile of the enzyme is independent from the optimal growth temperature of the host, likely representing a relic of evolution in high temperature environments. This finding is consistent with the hypothesis that mercury resistance evolved in thermophilic microbes. Work in progress is focused on the activity of the MR enzyme from an Archaeon.

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MICROBIAL INHABITANTS OF THE DEEP MARINE SUBSURFACE: STUDIES OF ODP LEG 201

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Little is known about the diversity and metabolic activities of microbes residing in the extreme environment of deep-sea sediment. This biosphere has become more accessible for microbiological study through the efforts of the Ocean Drilling Program (ODP). ODP Leg 201 traveled to the equatorial Pacific and Peru Margin to collect sediment cores for microbial analysis. Within these sediment cores, it is estimated that 10^8-10^5 cells/cm^3 exist in this cold habitat under high pressure. Our work on these sediments focuses on the cultivation of prokaryotes and the direct in-situ method of single cell analysis called FISH-SIMS. We are especially interested in cultivating psychrophilic microorganisms and have obtained a library of isolates from sediment depths up to 100 mbsf. Our isolates represent a number of genera and varied metabolic potential. Our isolations have been successful in rich marine media, low nutrient-low salt media, and even marine salts with no added carbon. Most isolates can grow over a wide temperature range, 2-30°C, and in a number of media types; both abilities that may allow continued survival as they are buried in the sediment column. In addition to examining these pure cultures in the laboratory, we are also analyzing single cells in fixed sediment cores. These fixed cores allowed us to perform direct single cell analysis to determine the type and metabolic activity of cells found in this deep-sea sediment. Cell type is determined by Fluorescent In-Situ Hybridization (FISH) and cellular metabolic activity can be inferred by analysis of natural ratios of carbon isotopes ($^{12}C/^{13}C$) using Secondary Ion Mass Spectrometry (SIMS). The combination of these techniques provides both a phylogenetic identification and carbon isotope ratio on a single cell from this sediment. Currently, we are examining areas of the sediment column which are expected to contain anaerobic methane oxidizing microorganisms. Methane in this environment is extremely light (-78 ‰), however, we have not yet found cells showing this light signature. In areas of increased cell counts and decreased methane, the archaeal cells examined by this technique have a heavier isotopic signature (approximately -20 ‰) indicating that these cells may be oxidizing methane, but not consuming it as cellular carbon. This environment contains many novel microorganisms, and may challenge our current ideas of metabolic activity over geologic time in the deep subsurface.

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MICROBIAL DIVERSITY IN THERMAL SPRING WATER AND ON COLONISED SURFACES

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In order to examine colonisation within terrestrial hydrothermal springs, glass slides have been immersed in high temperature geothermal springs and colonisation followed by molecular and microscopic methods. In New Zealand springs there was a surprising lack of species diversity as indicated by DGGE, morphology and culture methodologies in all pools investigated. Distinct DGGE profiles were obtained for the pH ranges 5.0-7.0 and 7.0-9.0 with commonly less than three dominant organisms present in each pH range. In pools above pH 7.0 a rod shaped organism was ubiquitous and dominant and was identified by 16S rDNA sequence and culture methods to be a member of the *Pyrobaculum* genus. This species had previously been isolated by conventional enrichment culture and characterised. Another organism present in the pools above pH 7.0 is a coccus. In many previous studies *Desulfurococcus* has been isolated from these pools by culture enrichment, however, slides in some of the pools studied are colonised by a coccus with a novel DGGE profile and 16S rDNA sequence. Obtaining pure cultures of this new organism has been difficult because it forms stable co-cultures with *Pyrobaculum*. A pure culture from one pool indicates the coccus grows well on konjac glucomannan and a limited range of carbohydrates. On the basis of 16S rDNA sequence and physiological characteristics we propose that these cocci constitute a new genus *Ignisphaera* within the crenarchaeota, with the type species being *Ignisphaera aggregans*.

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EFFECTS OF ENVIRONMENTAL FACTORS ON THE RESUMPTION OF PHOTOSYNTHESIS AFTER PROLONGED DESICCATION IN AN INTERTIDAL CYANOBACTERIAL MAT

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Much of the intertidal zone in the Laguna Ojo de Liebre, Baja California Sur is covered by Lyngbya-dominated microbial mats. The mats are desiccated for prolonged periods and are only hydrated during very high tides. When the water recedes and evaporation begins, the mats may experience extreme osmotic and temperature stress in addition to the ever-present stress of solar irradiance, including ultraviolet (UV). These extreme conditions also prevent the activity of invertebrate grazers, allowing the mats to spread throughout much of the intertidal. We conducted experiments to determine how certain environmental factors (e.g. salinity, light intensity and UV) affect the recovery of photosynthesis in these desiccated cyanobacterial mats immediately after rehydration. We used pulse amplitude modulated (PAM) fluorometry and carbon

incorporation assays to measure photosynthesis. Mats were rehydrated with 0-100ppt saltwater at 30°C under light intensities of 50 and 500 Wm-2, as well as in darkness. Using PAM fluorometry to measure photosystem II (PSII) efficiency, mats were found to recover within 30-60 minutes under optimal conditions. The shortest delays in photosynthetic initiation and the quickest overall recovery rates occurred in mats treated with 25 or 50 ppt saltwater under high light intensities (i.e. 500 W m-2). Both extremely high and low salinities were inhibitory with no recovery of PSII in fresh water. This is consistent with natural seawater coverage in this lagoon being optimal for recovery. Mats are exposed to salinities at or above 75 ppt only during dehydration, but are exposed seasonally to rain. Maximum photosynthetic carbon incorporation rates were attained in about 60 minutes. Consistent with PSII efficiency results, high salinities were inhibitory to carbon incorporation as well. However, the highest rates of carbon incorporation were found in mats rehydrated with fresh water. This discrepancy in the results may be attributed to some dissimilarity between the two techniques and not to physiological differences. UV did not appear to affect the recovery of photosynthesis. This is probably because the surficial Lyngbya filaments contained a high content of the UV screening compound, scytonemin, in their sheaths. It is interesting that even though these cyanobacteria spend the vast majority of their existence in the desiccated state or in suboptimal growth conditions, they are not only able to maintain mat structure, but expand and rapidly colonize exposed substrate.

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THE *GLOBULOVIRIDAE* AND THE *BICAUDAVIRIDAE*, NOVEL FAMILIES OF CRENARCHAEAL VIRUSES

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We will present description of two novel crenarchaeal viruses, *Pyrobaculum* Spherical Virus (PSV), isolated from Obsidian Pool in Yellowstone National Park, and *Acidianus* Two-tailed virus (ATV), isolated from an acidic hot spring in Pozzuoli, Italy. Unique morphotypes and genome organisation of viruses necessitated introduction of novel virus families for their classification, the *Globuloviridae* for PSV, and *Bicaudaviridae* for ATV. PSV is the first virus to be described which infects archaea of the genus *Pyrobaculum*. It replicates also in *Thermoproteus tenax*. The virus is not lytic and persists in its hosts in a carrier state. Spherical virions, about 100 nm in diameter, are enveloped and contain a major multimeric 33kDa protein and host derived lipids. A viral envelope encases a superhelical nucleoprotein core containing linear double-stranded DNA of 26 837 bp. The genome is unique for known archaeal viruses in that none of the genes show any significant matches to genes in public databases. Exceptionally for an archaeal virus, almost all recognisable genes are located on one DNA strand. ATV, infecting the hyperthermophilic archaeon "*Acidianus convivator*", has the exceptional property of undergoing a major morphological development extracellularly. Virions are extruded from host cells as lemon-shaped particles, and, thereafter, they develop long tails at each pointed end at temperatures above 75°C. Infection with ATV results either in viral replication and subsequent cell lysis or conversion of the infected cell into a lysogen. In lysogens the virus-encoded integrase facilitates integration of the viral genome into the host chromosome. Lysogeny can be interrupted by different stress factors. The virions carry a protein envelope and at least eleven proteins with molecular masses in the range of 12-90 kDa. The circular double-stranded DNA genome, 62730 bp long, was sequenced. Four IS elements are present in the genome. Several ORFs encode polypeptides with similarities to proteins of eukaryal viruses involved in interactions with hosts.

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INVESTIGATION OF ARCHAEAL COMMUNITY BY 16S RRNA AND FLUORESCENCE *IN SITU* HYBRIDIZATION IN SULFURIC HYDROTHERMAL HOT SPRING, NORTHERN TAIWAN

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The archaeal community composition of Yangmingshan National Park in northern Taiwan was investigated via 16S rRNA and fluorescence in situ hybridization (FISH). Optimization of tetrameric restriction enzyme (TRE) was performed to achieve efficient digestion and differentiation in the restriction fragment length polymorphism (RFLP) fragments, and *Aci*I, *Bst*UI and *Rsa*I were shown to be the optimal TREs for TRE-RFLP. Nine clones were obtained in the studies, with clones M70 (genbank accession no. AY627864) and M6 (genbank accession no. AY627858) being found to be phylogenetically affiliated to *Sulfolobus* and *Caldisphaera* in domain Crenarchaeota, respectively, whereas seven other clones were found to be affiliated to uncultured and unidentified archaeon isolated from thermoacidic environments. In FISH, soil and water region cells were hybridized with DAPI (4', 6-diamidino-2-phenylindole) and specific fluorescently labeled probes. 15.69 % and 7.16 % of the DAPI-stained cells hybridized with archaeal probe ARC915 and sulfate-reducing bacterial probe SRB385, respectively.

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PACIFIC NORTHWEST ALPINE GLACIER MICROBIAL COMMUNITIES

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Glacier microbes offer high potential for community biodiversity, evolutionary research, as climate and pollution indicators, and in biotechnological applications, especially through adaptations to freeze-thaw and high ultra-violet light. In the Pacific Northwest of North America, glaciers supply up to one third streamflow (with an undoubtedly a significant biological contribution) during the dry season, required for Pacific salmon and other downstream

inhabitants. This contribution, physical and biological, is threatened, as PNW glacier are experiencing an average terminal retreat greater than 25 m per year, an average volume loss of 4% per year. Although some cryobionts have been described, freshwater glacier communities remain relatively undocumented. We have collected and enumerated microbial and macroinvertebrate surface and near-surface samples from 25+ glaciers in Montana, Alberta, British Columbia, Alaska, Washington and Oregon, over a period of several years. Graduate and undergraduate students perform module research, as part of a long-term study, in cooperation with experts in numerous fields. Maximum surface densities appear in mid-afternoon on the sunniest days (~45,000 cell/ml in snow on glaciers), so mechanisms to deal with intense UV levels must be very effective. Higher densities were also associated with higher elevations ($p<0.001$), suggesting a greater contribution of the physical attributes of snow (compared to ice and liquid water) than previously believed. This trend is supported by highest average microbial densities in snow runnels (~80,000), seconded by average stream densities (~65,000), with average ice and pool water densities much lower (<5,000). Macroinvertebrate densities are positively correlated with the microbial component. A glacier with an outflow of 28 m^3/second, contributes over 42 billion microorganisms *per second* downstream in the summer. These previously undocumented communities include previously identified, newly identified, and unidentified taxa. Greater expertise is needed for morphological evaluation; molecular identification will begin this year. Collections of PNW glacier biofilm and pool substrate collections have been made, but have not been analyzed, and are a high research priority. Assistance from the microbial scientific community is needed. The rate of PNW glacier retreat advocates greater urgency in the study of these communities.

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ANHYDROPHILIC, HALOTOLERANT MICROBIAL MATS OF SAN SALVADOR, BAHAMAS

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Like many Bahamian Islands, San Salvador Island (24°05' N, 74°30' W) contains numerous shallow, hypersaline (45 to 322 ‰) lakes. The lakes are subjected to intense irradiance (> 2100 iE m-2 s-1), high temperatures (> 35° C) and chronic nutrient depletion. Highly productive microbial mats blanket the shallow sediments in many of the lakes. The overall research objective of this study is to assess the influence water availability has on structural diversification, community composition, production, and carbon sequestration in microbial mats. Three transects, 26 meters in length, have been established along a natural desiccation gradient in one of the hypersaline lakes, Salt Pond. Samples for community composition, extracellular polymeric substances (EPS) content, C & N content, and microscopic documentation are collected during each site visit (two to three times a year). Rates of key C, O, and N cycling processes (photosynthesis and N_2 fixation) are obtained. In cooperation with the staff from the Gerace Research Center, Salt Pond's salinity and temperature are being measured every 10-21 days. From March to July, Salt Pond's salinity increased from ~ 110‰ to over 320‰. Light and dark vertical O_2 distribution profiles of the mat's upper 5 mm indicate that, under dark conditions, anoxia reaches the mat surface. When exposed to light (1,500 µmol m^{-2} s^{-1}, 10 min), O_2 was detected as deep as 5 mm with concentrations (ca. 800% O_2 saturation) peaking at 1 mm depth. Light and dark cycles create a dynamic chemical environment that changes from anoxic to hyperoxic conditions within minutes. How EPS may buffer against drastic changes in redox conditions is being examined. Nutrient addition bioassays (e.g., NH_4^+, NO_3^-, and PO_4^{2-}) indicate salinity levels and not nutrient availability has the greatest impact on these crucial biogeochemical processes. Sequencing surveys of cyanobacterial 16S (primary producers), *dsr* (sulfate reducers/carbon mineralizers), and *nifH* (diazotrophs) genes show that diverse assemblages comprise the key functional groups of microorganisms. Carbohydrate analyses have led to the discovery of "amadori products" (APs) in the Salt Pond mats. APs are unique protein-carbohydrate linkages that form when basic amino acids cross-link with carbohydrate carboxyl groups. Bacterial isolates from the mats and lake water include gram positives, alpha and gamma proteobacteria, and flavobacteria. Currently, we are attempting to isolate non-sporulating organisms that are desiccation tolerant.

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INTRA- AND INTERSPECIES INTERACTIONS IN CO-CULTURES OF THE HYPERTHERMOPHILIC BACTERIUM *THERMOTOGA MARITIMA* AND ARCHAEON *METHANOCOCCUS JANNASCHII*

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Microbial interactions in high temperature environments have not been well characterized to this point, although such relationships form the basis of the ecology in these niches. In this study, both pure and co-cultures of model hyperthermophilic microorganisms, *Methanococcus jannaschii* and *Thermotoga maritima*, have been used to explore high-temperature microbial ecology from several perspectives. When grown in co-culture, the archaeon *M. jannaschii*, an autotrophic methanogen, was found to utilize hydrogen and carbon dioxide, produced by the fermentative bacterium *T. maritima*. In turn, *T. maritima* benefited from the presence of the methanogen due to the removal of inhibitory extracellular hydrogen, resulting in a four-fold increase in *T. maritima* maximum cell density over growth in pure culture. A full-genome cDNA microarray for *T. maritima* was constructed and used to examine gene expression during log phase in replicate cultures containing *T. maritima* monocultures, with and without mechanical hydrogen removal, and in co-cultures with *M. jannaschii* effecting hydrogen removal. Transcriptional response was analyzed using two separate ANOVA mixed models, a global normalization model and a gene-specific model. Results indicate that bottlenecks related to hydrogen inhibition were alleviated by

either co-cultivation or mechanical sparging. However, significant differences were noted in transcriptional response data in co-culture. Notably, it was found that cyclic di-GMP intracellular signaling via GGDEF-domain proteins was apparently involved in the regulation of exopolysaccharide production in high cell density co-cultures. While quorum sensing controls global regulation of the biofilm phenotype in many mesophilic bacteria, this phenomenon has yet to be found in hyperthermophiles. To determine if quorum sensing may be the global regulator controlling the switch to the biofilm lifestyle at high cell density, composition of extracellular small molecules in cultures of *T. maritima* was examined using high-throughput 2-D mass spectrometer analysis. Subsequently, putative signal molecules identified via this technique were produced synthetically and analyzed for biological activity in pure and co-cultures using the full genome microarray to measure the transcriptional response to the putative signal molecules. This study suggests that higher order interactions in hydrothermal environments merit additional attention and that functional genomics approaches in mixed cultures are plausible for such investigations.

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COMMUNITY DIVERSITY OF PROKARYOTES IN TIBETAN GEOTHERMAL SPRINGS

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A survey of 16S rDNA-defined community diversity was carried out for microbial mats and streamers in the Daggyai Tso geothermal springs in Tibet, China. These near-neutral pH, low sulphide springs represent one of the most isolated habitats for thermophiles due to their high altitude (~5,500m) and geographic isolation. Mats were collected from pools of 52-69°C, and streamers from pools of 65-83°C, during a field-sampling season in summer 2003. Community DNA was amplified using domain-specific primers for eubacteria and archaea, and separated by denaturing gradient gel electrophoresis (DGGE). Bands were excised and sequenced, and matches to Genbank sequences of thermophilic organisms from other locations determined. Mats were comprised of the oxygenic phototroph *Synechococcus*, with several distinct genotypes recovered. Other sequences shared high similarity to the green nonsulphur bacteria genus *Chloroflexus*, and several non-photosynthetic genera. Streamers from 65°C and 70°C pools supported photosynthetic and non-photosynthetic genera, whilst those from the 83°C pool supported a restricted community of non-photosynthetic bacteria. Sequence data is discussed in terms of community structure with regard to temperature, and the effects of geographical isolation on diversity.

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MICROBIAL COMMUNITY UNDER THE HYDROTHERMAL SYSTEM ON THE SUIYO SEAMOUNT

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Microbial community in hydrothermal area at seafloor has been analyzed by culture-independent methods. Hydrothermal fluid from natural vents and vent chimneys have been analyzed by PCR (1-2). Hyperthermophilic microbes have been isolated from these environments (3-4). Though the analysis of these samples can provide the window to penetrate the microbial community under the seafloor, more direct analysis is desired for better understanding of the sub-seafloor microbial community. In the "Archaean Park Project" supported by Special Coordination Fund, several holes were drilled and the holes were supported by casing pipes in the crater of the Suiyo seamount on the Izu-Bonin arc, West Pacific Ocean (about 1,400 m depth) in 2001 and 2002. Hydrothermal fluids were sampled from cased holes. The fluids were filtered to collect the microbial cells. The DNA was extracted and used to amplify 16S rDNA fragments by PCR using a bacteria and an archaea specific primer sets. The PCR fragments were cloned and sequenced. FISH analysis revealed from 6 x10**3 to 2.5 x 10**6 bactrerial cells/ml in these hydrothermal fluids. PCR clone-analysis showed significant variation in bacterial sequences found in these samples. The species-patterns suggest that the contamination of ambient seawater to hydrothermal fluid samples is negligible. Difference in the dominant species depending on the location was found, suggesting that the bacterial community at sub-sea floor is not monotonous but has gradual shift from the hydrothermal center to peripheral area. The results suggest that there is chemo-autotrophic microbe-dependent biota under the hydrothermal system.

1) Takai et al. Genetics 152: 1285-1297 (1999)
2) Takai et al. Appl. Environ. Microbioi. 67: 3618-3629 (2001)
3) Summit et al. Proc. Natl. Acad. Sci. 98: 2158-2163 (2001)
4) Amend, J. P. and Shodk, E. L. FEMS Microbiol. Rev. 25: 175-243 (2002)

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USE OF CONTINUOUS CULTURE TO ACCESS THE CULTIVABLE THERMOPHILIC MICROBIAL DIVERSITY FROM DEEP-SEA HYDROTHERMAL VENT

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Since the discovery of deep-sea hydrothermal vents, microbial diversity of these extreme environment was studied using both cultivation techniques and more recently molecular techniques. Cultivation techniques (with great effort focused on thermophily) have led to the isolation and characterisation of a large number of new species belonging to both archaeal and bacterial kingdoms and involved in various geo-biochemical cycles since they use a large panel of electron donors and acceptors. Molecular diversity studies

mainly based on 16S rDNA sequences analysis give us a picture of what is present in the ecosystem but do not permit to infer a physiological role of micro-organisms in their environment when the retrieved sequences are related to phyla that do not include cultivated representatives and also do not take in account the phenotypic diversity within already known groups. Since current culture techniques do not always provide a balanced picture of the microflora composition, development of new culture methods is needed to approach as closely as possible the conditions of natural habitats with emphasis of cultivation of microbial communities instead of pure culture. Enrichment cultures were performed from hydrothermal vent chimney sample using a continuous culture in a gas lift bioreactor,.(initially developed for the cultivation of hyperthermophilic anaerobic micro-organisms).Two enrichment culture experiments in the gas lift bioreactor were conducted over several weeks at both 60°C and 90°C and the microbial diversity were analysed using molecular tools. Cloning and sequencing of 16S rRNA genes of 2 samples of the 2 cultures was performed and a DGGE analysis of the 16S rDNA diversity was also performed all over the 2 cultures in order to monitor the dynamic of the microbial population. The results evidenced quite large diversity compared to previously obtained in the same conditions by classical batch cultures in closed vessels At 90°C, while archaeal diversity was limited to species belonging to the *Thermococcales*, an unexpected bacterial diversity was obtained including still uncultivated species and already described species growing at temperature over their previousely known optimal growth conditions .At 60°C, large bacterial diversity was achieved and both heterotrophic and autotrophic species were shown to be cultivated. These preliminary experiments let us conclude that such a bioreactor system might allow cultivation of thermophilic microbial communities from deep sea hydrothermal vent and allow the study of interaction between species in various experimental conditions.

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SPATIAL AND SEASONAL SURVEY OF MICROBIAL DIVERSITY OF SOAP LAKE, A MEROMICTIC SODA LAKE IN WASHINGTON

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Select anaerobic activities and the composition of members of the domain *Bacteria* in samples from alkaline, saline, and meromictic Soap Lake in Washington were studied. This lake is an extreme environment as the salinity ranges from 15 to 145 g/L and the pH ranges from 9.7 to 10.2 throughout the water column. Sediment pH values range from 10.1 to 11.3. Summer and fall samples were collected from the mixolimnion , the chemocline, the monimolimnion, and sediment. Most-probable number assays targeting anaerobic microbial guilds were established. In both seasons, the anoxic samples appeared to be dominated by lactate-oxidizing sulfate-reducing bacteria. Lactate- and acetate-oxidizing iron-reducing bacteria, as well as fermentative microorganisms, were also detected at high numbers, whereas methanogenic numbers were low or undetectable. In addition, community structure was assessed by sequencing cloned fragments of 16S rRNA genes retrieved from extracted DNA. Most summer sequences fell into five major lineages of the domain *Bacteria*: low G+C content gram-positive organisms (29%), *Cytophaga-Flexibacter-Bacteroides* (CFB, 19%), *Proteobacteria* (ã-, ä-, and á-, 5%, 7%, and 8%, respectively), *Synechococcus*-like cyanobacteria (6%), and *Chloroflexi* (6%). Populations of sequences from the mixolimnion were dominated by sequences related to ã-*Proteobacteria*, while those from the chemocline, the monimolimnion, and the monimolimnion sediment were dominated by sequences related to low G+C gram positive bacteria. The mixolimnion sediment was equally represented by sequences related to low G+C gram positive bacteria, *Chloroflexi*, and CFB. The majority of fall sequences fell into the following lineages of the *Bacteria*: CFB (25%), low G+C gram-positive organisms (21%), ã-*Proteobacteria* (17%), *Chloroflexi* (9%), and *Actinobacteria* (8%). Groups of sequences recovered from the mixolimnion (both 5 m and 15 m) were dominated by sequences related to ã-*Proteobacteria*, while the population of sequences obtained from the chemocline and the monimolimnion sediment were dominated by sequences related to low G+C gram-positive bacteria. The monimolimnion was dominated by sequences related to CFB, and the mixolimnion sediment was mainly represented by sequences distantly related to an uncultured group of *Chloroflexi*. The percentage of unique phylotypes relative to the number of sequences was, for both seasons, higher in the monimolimnion and the sediments than in the oxic, upper layers. Overall, the results suggest (*i*) that anaerobic activities represent an important contribution to carbon cycling, and (*ii*) that elevated diversity occurs in anoxic portions of the lake.

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MICROBIAL BIOGENESIS IN FERROMAGNETIC REDUCED MARTIAN SIMULANT SOIL, UNDER UV LIGHT: USE OF ELECTROSENSORS

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Microbial biogenesis studies in extreme environments in Earth and in extraterrestrial conditions such as in Mars have been difficult, because the lack of sensitive detection techniques, most studies have been done using dissimilar substrates for comparative experiments of microbial survival, and absence of an electrochemical-biological interactive approach. Thus, these parameters should simulate more realistically the initial extreme conditions in Earth and in other planets such as Mars in relation to microbial survival. Therefore, the aim of this study was to determine survival of low detectable autotrophic bacteria (*Acidithiobacillus ferroxidans*) in highly reduced Martian simulant soil in an electrolysis cell divided by a semipermeable cationic membrane, under different redox potentials, light spectrum including ultraviolet light, and different oxygen levels. Martian simulant soils were inoculated with the autotrophic iron-reducing bacterium *A. ferroxidans*, and incubated at room temperature. The electrolysis cells was covered by a lid with ports to insert different electrosensors including measurement and reference

electrodes, pH, temperature, and oxygen probes. The system was run by a measurement control interface (MCI), which was connected to a high-impedance galvanically isolated differential amplifier and to an analog-to-digital control card, and recorded by computer through an intelligent expert system. Different parameters were measured and correlated, electroconductivity, redox potential, oxygen levels, pH, ferromagnetism, iron concentration, cell growth, bacterial cell-iron interaction through transmission electron microscopy, and DNA/RNA expression. The results showed that the ratio between Fe^{+2}/Fe^{+3} determined the growth of the bacterium *A. ferroxidans* in the Martian simulant soil. However, the soil ferromagnetism along with the presence of UV light influenced both growth and survival of the bacteria in the Martian simulant soil. UV light stimulated bacterial growth in higher ferric soil. Electron emission correlated with growth of the bacteria, under the conditions of the investigation. Further investigation is underway.

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MICROBIAL DIVERSITY OF THE BACTERIAL MAT SEDIMENTS IN THE EASTERN MARGIN OF JAPAN SEA

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The eastern margin of the Japan Sea is believed to be a convergent plate boundary between the Amurian and Okhotsk plates. The bacterial mats were found at the eastern wall of the Shiribeshi Trough in the Japan Sea at a depth of 3,145 m in 1999. The existence of these bacterial mats might be by cold-seepage oozes out in a wide area through the high gravel of preciousness. In this study, we investigated the microbial diversity of the bacterial mat sediments obtained from the Japan Sea. We re-visited those bacterial mats site in 2003. Using the manned submersible SHINKAI 6500, bacterial mat sediments and push-core sediments were collected at a depth of about 3,000 m in the Japan Sea. The push-core sediment was collected each 1 cm length. Using the sediment DNA isolation kit, the DNAs were extracted directly from the each sediment sample. Bacterial or Archaeal 16S rDNAs for terminal restriction fragment length polymorphisms (t-RFLP) analysis were amplified by the polymerase chain reaction (PCR). The sequences of the amplified 16S rDNAs were also determined and compared with sequences in the DNA databases. The PCR amplifications of sulfite reductase genes from push-core sediment samples were also performed. From the t-RFLP analysis, the differences of the 16S digested patterns between the bacterial mat sediments were not observed. In the push-core sediment samples, gamma-Proteobacteria were decreased and anoxic methane oxidizing archaea and Methanogens were increased in a vertical direction. From the sequencing and phylogenetic results, the mat sediment samples were included abundant delta-Proteobacteria, which are related to sulfate-reducing bacteria, epsilon-Proteobacteria and Methanogens including the anoxic methane oxidizing archaea. Moreover, some relatives of sulfur-oxidizing bacteria were also observed in the mat samples. Sulfite reductase genes were identified from push-core sediment samples from 2 to 8 cm depth. Bacterial mat sediment samples and push-core sediment samples of from 0 to 2 cm and from 8 to 23 cm were not identified the sulfite reductase genes. It was indicated that gamma-Proteobacteria inhabit in surface, sulfate-reducing bacteria inhabit from 2 to 14 cm (probably together with the anoxic methane oxidizing archaea) and Methanogens inhabit deeper than 8 cm.

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LIVING MICROBES ON SILT GRAINS IN GREENLAND BASAL ICE AT 3053 M DEPTH

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Two ice cores 30 km apart extended ~3050 m down to bedrock at Summit, Greenland. Most attention has been focused on their high-resolution climate record covering the last ~110,000 years. In the bottom few meters, which contained up to 0.65 wt % of silt (mean diameter 3 μm), R. Souchez *et al.* found a factor of 500 excess of CO_2 and a factor of 10^4 excess of CH_4 in the GRIP basal ice, and T. Sowers measured a factor 2 x 10^4 excess of CH_4 at 3043 m in the nearby GISP2 basal ice. V. Miteva *et al.* discovered ~7 x 10^7 microbial cells/ml in Sowers' sample. Souchez *et al.* inferred from the huge excesses that the ice formed by mixing with a peat deposit. It is now clear from our work and the observations of Miteva *et al.* that the excesses were the direct products of *in-situ* microbial metabolism. The basal ice, with its silt and microbial population, likely formed before the ice sheet developed. Using DAPI stain and epifluorescence, we analyzed the relationships of microbes to silt grains at six depths from 3044 to 3053 m in GISP2 ice. Microbial concentrations ranged from ~10^7 to >$10^9/cm^3$ of ice and correlated with weight fraction of silt rather than with height. Between 92% and 99% of the microbes were attached to silt particles at a typical concentration of ~$10^7/cm^2$ of grain surface. This correlation extended down to the smallest silt grains: typically, up to 10 cells were attached to a 2 μm grain. With an SEM we found that some cells were dividing, and we were able to culture microbes in low-nutrient liquid media. These two results demonstrated their viability after 10^5 yr at -9°C.

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MOTPS IS THE STATOR-FORCE GENERATOR FOR MOTILITY OF ALKALIPHILIC *BACILLUS* AND ITS HOMOLOGUE IS A SECOND FUNCTIONAL MOT IN *BACILLUS SUBTILIS*

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The stator-force generator that drives Na^+-dependent motility in alkaliphilic *Bacillus pseudofirmus* OF4 is here identified as *motPS*, a *motAB*-like pair of genes that are downstream of the *ccpA* gene, which encodes a major regulator of carbon metabolism. Motility of

B. pseudofirmus OF4 was only observed at pH values above 8, was most strongly displayed on soft agar surfaces and was also observed in liquid. Disruption of *motPS* resulted in a non-motile phenotype that was corrected by restoration of the genes on a multi-copy plasmid. The purified and reconstituted MotPS from *B. pseudofirmus* OF4 catalyzes amiloride analogue-sensitive Na^+ translocation. The role of the homologous *motPS* genes from *Bacillus subtilis* in several motility-based behaviors was also tested in isogenic strains expressing both *motAB* and *motPS*, only one of the two *mot* systems, or neither *mot* system. *B. subtilis* MotPS (BsMotPS) supported Na^+-stimulated motility and chemotaxis on soft agar surfaces as well as biofilm formation, especially after selection of an up-motile variant. BsMotPS supported only modest motility in liquid and did not support surfactin-dependent swarming on higher concentration agar surfaces. Differences between the wild-type and *motPS* mutant strains indicated that BsMotPS contributes to biofilm formation and motility on soft agar, but not to swarming, in cells containing a functional MotAB.

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MOLECULAR SPECIES COMPOSITION OF ARCHAETIDYLCHOLINE FROM THE HYPERTHERMOPHILIC METHANOARCHAEON METHANOPYRUS KANDLERI

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A choline-containing phospholipid (PL-4) in Methanopyrus kandleri cells was identified as archaetidylcholine by positive staining with Dragendorff-reagent on a TLC plate and FAB-mass spectrometry. PL-4 consisted of a variety of molecular species that were different in hydrocarbon composition. Most of PL-4 was acid-labile because of its allyl ether bond. A method of LiAlH4 hydrogenolysis was used for cleavage of allyl ether bonds and recovery of the corresponding hydrocarbons. Four kinds of hydrocarbons formed were identified by gas-liquid chromatography and mass spectrometry. The composition of the hydrocarbon species of PL-4 was estimated by quantitative hydrogenolysis with LiAlH4 as hydrocarbon with four double bonds, 47%; three double bonds, 11%; two double bonds, 14%; and one double bond, 7%. Hydrocarbons of PL-4 obtained by HI cleavage and subsequent reduction were saturated (6%) and non-allylic unsaturated (16%) species. The molecular species composition of PL-4 was also estimated by its acid lability. About 77% of PL-4 phosphate changed to water-soluble products (glycerophosphocholine or glycerophosphate), which were assumed as allyl-allyl molecular species. Four major spots were detected on TLC of the chloroform-soluble fraction obtained after HCl-degradation. Unchanged archaetidylcholine and archaetidic acid (the non-allyl-non-allyl ether molecular species), and lysoarchaetidylcholine and lysoarchaetidic acid (allyl-non-allyl molecular species) were identified by their mobility on TLC. Archaetidic acid and lysoarchaetidic acid were by-products formed by removal of choline during acid treatment. Phosphate contents of allyl-non-allyl ether molecular species were 11%. Non-allyl-non-allyl molecular species accounted for 11%. The total percentage of four kinds of allyl ether species was 78 %, which coincided well with 83 % allyl ether type PL-4 based on the acid lability. Considering the mechanisms for isoprenoid and ether phospholipid biosynthesis, the hydrocarbon chain of PL-4 with four double bonds is most likely a geranylgeranyl chain. Assuming that the double bonds are located at the position 2, 6, 10, and/or 14, there must be 16 different molecular species with zero to four double bonds randomly at the four positions. Because archaetidylcholine has two hydrocarbon chains in one molecule, 256 molecular species are possible, of which 64 are allyl-allyl ether types.

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CHARACTERIZATION OF A THERMOSTABLE SULFUR OXYGENASE/REDUCTASE FROM THE THERMOACIDOPHILIC ARCHAEON ACIDIANUS SP. S5

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Oxidation of sulfur is a typical energy-yielding mechanism among species of Sulfolobus, Thiobacillus and Acidianus. The extremely thermoacidophilic archaeal genus Acidianus is capable of oxidation and/or reduction of sulfur, to obtain energy for growth. When cultivated aerobically, cells grow by oxidation of sulfur or hydrogen sulfide to sulfate. When cultivated anaerobically and in the presence of molecular hydrogen, cells grow by reduction of sulfur to hydrogen sulfide. In Acidianus species, two types of enzymes involved in sulfur oxidation have been described: The sulfur oxygenase (SO) from A. brierleyi oxidizes sulfur to sulfite, and formation of hydrogen sulfide has not been detected. However, in the absence of molecular hydrogen the SO/reductase (SOR) from A. ambivalens produces hydrogen sulfide simultaneously with sulfite during oxidation of sulfur. The biological significance of SOR remains questionable. Its role in energy metabolism is not clear since a link to the electron transport chain has not yet described. Although the SOR gene (sor) of A. ambivalens has been characterized at a molecular level, successful expression of active SOR in Escherichia coli has not been reported. In this report, we present the cloning, sequencing and overexpression. A thermoacidophilic, obligately chemolithotrophic, facultatively aerobic archaebacterium, Acidianus sp. S5, was isolated from acidothermal springs in southwest China. The sulfur oxygenase/reductase (SOR) gene of Acidianus sp. S5 was cloned and expressed in Escherichia coli. Several primers were designed and successfully applied for detection and cloning of the sor gene. A 3.7-kb EcoRI fragment containing the sor gene and three neighboring open reading frames was sequenced. Sequence analysis indicated that the sor gene of Acidianus sp. S5 showed 81% identity to the sor gene of Acidianus ambivalens. E. coli cells carrying the sor gene on pBV220SOR were able to overproduce SOR upon a temperature shift from 30 to 42C. SOR produced in E. coli catalyzes the oxidation of elemental sulfur and concomitant production of sulfite, thiosulfate and hydrogen sulfide. The recombinant enzyme exhibits the same catalytic properties as the one from Acidianus S5. As far as we now, this is the first report of a successful expression of a sor gene from a thermoacidophilic archaeon in E. coli. Overproduction of SOR in

E. coli may facilitate future studies of this enzyme. As we have demonstrated, SOR expressed in E. coli can easily be recovered by heating cellular lysates and precipitation of denatured contaminating proteins. The high level production of SOR will further facilitate the purification of the enzyme.
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CHARACTERIZATION OF A SOUBLE FERRIC REDUCTASE(S) FROM THE THERMOPHILIC BACTERIUM *THERMOTERRABACTERIUM FERRIREDUCENS*

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Microbially-mediated Fe^{3+} (iron (III)) reduction has only fairly recently been recognized as one of the most important processes in anaerobic soil. Iron is the fourth most abundant element on earth but is not readily available for biological use since it has a low solubility (10^{-18}) at a neutral pH. This fact makes evolved biological mechanisms for the acquisition of iron particularly interesting. Presently, the majority of described Fe^{3+}-reducers are mesophilic, Gram-type negative Proteobacteria. More recently, several thermophilic Fe^{3+}-reducers have been described. *Thermoterrabacterium ferrireducens*, a Gram-type positive, Fe^{3+}-reducing anaerobic thermophile (optimum growth temperature 65°C), is thought to have a Fe^{3+} reducing activity linked to the cell membrane. *T. ferrireducens* cell lysate was partitioned into membrane and soluble (cytoplasmic) fractions by ultra-centrifugation at 105,000xg. Fe^{3+} reduction to Fe^{2+} was monitored by the use of the colorimetric Fe^{2+} capture reagent ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine). Maximal activity was obtained when the assay included: 40 mM $MgCl_2$, 1.0 mM NADH, 4.0 µM horse heart cytochrome c and 1.0mM riboflavin. Enzymatic Fe^{3+} reduction was found to be equally localized to the membrane and soluble fractions of *T. ferrireducens*. Activity in both the soluble and membrane fractions was enhanced by the addition of 1.0 mM FMN (flavin mononucleotide) or 1.0 mM riboflavin (but not 1.0 mM FAD) to the activity assay. Fractions with high Fe^{3+} reduction rates of 58 nmol/min/mg protein (at 60°C) were obtained from the soluble cell fraction by the use of: a strong anion exchanger (Q-Sepharose fast flow media), an affinity column (Hi Trap Blue Sepharose) and gel filtration (Sephacryl S-300 HR). *T. ferrireducens* has active Fe^{3+} reductase activities localized to both the membrane and soluble cell fractions. Purification of the Fe^{3+} reductase has been obtained from the soluble cell fraction. The difference between the membrane-bound and the soluble Fe^{3+} reductase are presently unknown.

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TRANSCRIPTION AND TRANSLATIONAL ANALYSIS OF THE ALCOHOL METABOLISM OF *SULFOLOBUS SOLFATARICUS* P2

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Prior exploration has generated information on metabolic pathways, with transcriptomic and proteomic studies facilitated by the recent genomic sequencing of *Sulfolobus solfataricus*, P2. In this study, focus has concentrated on the products of alcohol metabolism. Here, we sought to analyse the transcriptional and translational response of *S. solfataricus* to various types of alcohols. *S. solfataricus* P2 was grown at 80°C in Basal medium, supplemented with 0.4% w/v of glucose and 0.5-1.0% v/v of either 1-propanol, 2-propanol (iso-propanol), ethanol, phenol or acetone. Differences in protein expression were analysed using 2-D PAGE. Proteins were visualised with large format gels (17 cm) using both broad (pH 3-10) and narrow range (pH 6-10) IPG strips. Over 400 spots were generated per phenotype, with differential changes assessed using Bio-Rad's PDQuest software. Gels were mapped to allow for a better understanding of translational correlations within various phenotypes. Analysis of trypsin-generated peptides from differentially expressed proteins was undertaken using electrospray ionization quadrupole time-of-flight tandem mass spectrometry equipped with a capillary liquid chromatograph. Peptide mass fingerprints from MS and MS-MS studies were analysed using Mascot and Pro ID software against the complete National Centre for Biotechnology Information non-redundant protein database. RT-PCR was carried out using primers specifically targeting the 14 putative alcohol dehydrogenase (ADH) genes in *S. solfataricus* to identify the functionality of ADH genes (presence, absence and intensity). These results allow for alcohol pathway reconstruction within this organism, and ought to facilitate future ADH metabolic engineering strategies.

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SEASONAL CHANGE AND CULTURABILITY OF BACTERIAL COMMUNITIES IN A SUB-ARCTIC LAKE

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Heterotrophic bacteria remineralize the organic compounds from primary production to sustain food webs in aquatic systems, reduce the concentrations of dissolved organics, and produce major quantities of carbon dioxide, a green house gas. To understand the equilibrium between dissolved organics and heterotrophic aquatic bacteria and species distribution over the annual cycle in a subarctic lake - Harding Lake, Fairbanks, Alaska, we used multiple approaches for the investigation, such as terminal restriction fragment length polymorphism (TRFLP), denaturing gradient gel electrophoresis (DGGE), flow cytometry, microscopy, and DNA sequencing. We found that there was a near complete change of species while the

bacterial content of Harding Lake water was relatively constant over the seasons. Sequencing of the 16S rDNA DGGE bands, together with phylogenetic analyses, suggested that the dominant bacteria from three seasons were mainly comprised of á-proteobacteria-, ã-proteobacteria-, cyanobacteria-, and actinobacteria-related phylotypes. Using the extinction culture protocol, there was an apparent decrease in culturability with inoculum size according to populations detected by epifluorescence microscopy, flow cytometry, and TRFLP. Extinction cultures were perpetuated for extended periods in unamended, filtered, autoclaved lake water. Specific affinities for amino acids were only 20 to 1500 L/g-cells hr, winter vs summer, requiring simultaneous use of numerous substrates. We conclude that bacterial growth rate was sufficient for complete population change, even in winter when uptake nutrient rate was very small. Therefore, winter populations must be able to use many substrates simultaneously. Further, most species compete for the same substrate group leading to a small number of dominant species. Apparent culturability approached 10% in unamended lake water, but decreased with inoculum size.

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BACTERIAL GROWTH ON CRUDE OIL AND OIL COMPONENTS UNDER SULFATE REDUCING CONDITIONS

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Oil reservoirs in The North Sea are situated 1,6 to 6 kilometres below the seabed. The reservoir temperatures range from 60 to 200°C, and the pressure from 15 to 80 MPa. The oil reservoirs are extreme environments, characterized by a combination of high temperature and pressure, high concentrations of recalcitrant organic carbon, absence of O_2, and low concentrations of other electron acceptors. Such environments should hardly be expected to support life, but nevertheless prokaryotic organisms are present at this "life on the edge". Seawater injection is often used to retain the reservoir pressure during oil production. The seawater cools the rock- and water formations near the injection site and makes the temperature conditions more suitable for microbial life. Crude oil and oil components in the reservoir and sulfate from seawater provide nutrients for hydrocarbon oxidising sulfate reducing bacteria (SRB). The end product of sulfate reduction is hydrogen sulfide (H_2S). H_2S production in oil reservoirs (reservoir souring) is of great concern to the oil companies. H_2S is a highly toxic gas that causes corrosion of iron and steel alloys, and decreases the quality of the oil and gas taken out of the reservoirs. The gas is also a health hazard to the oil field workers. SRB degrade aliphatic and aromatic hydrocarbons by a radical mechanism, different from aerobic hydrocarbon degraders [1-4]. So far however, little is known about microbial oil degradation in oil reservoirs. By using hydrocarbon-degrading SRB-systems, including an oil reservoir model column [5] and SRB batch cultures, we study factors involved in microbial oil degradation and H_2S production. The primary goal is to understand factors that control H_2S production in oil reservoirs. Here we present some preliminary results from the SRB-isolation- and characterization process. The work is a collaboration project with the Department of Chemistry, University of Bergen, Norway.

References: [1] Rabus, R., Heider, J. (1998). Arch. Microbiol. 170:377-384. [2] Spormann, A.M., Widdel, F. (2000). Biodegradation 11:85-105. [3] Widdel, F., Rabus, R. (2001). Curr. Opinion. Biotechnol. 12:259-276. [4] Wilkes, H., Rabus, R., Fischer, T., Armstroff, A., Behrends, A., Widdel, F. (2002). Arch. Microbiol. 177:235-243. [5] Myhr, S., Lillebø, B.-L.P., Sunde E., Beeder, J., Torsvik, T. (2002). Appl. Microbiol. Biotechnol. 58:400-408.

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SSPA, AN OUTER MEMBRANE PROTEIN, IS HIGHLY INDUCED UNDER SALT-STRESSED CONDITIONS AND ESSENTIAL FOR SALT-STRESSED AEROBIC GROWTH CONDITIONS IN *RHODOBACTER SPHAEROIDES* F. SP. *DENITRIFICANS* IL106

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SspA, an outer membrane protein, is highly induced under salt-stressed conditions and essential for salt-stressed aerobic growth conditions in *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106 *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106 (R. *sphaeroides*) was initially isolated from freshwater, but adapts up to 4% (0.68 M) NaCl in the culture medium. In the course of our study on salt-tolerance mechanism of *R. sphaeroides*, we demonstrated that trehalose was accumulated as osmoprotectant in the cytoplasm and a novel outer membrane protein, SspA, was remarkably increased by means two-dimensional electrophoresis analysis. SspA displays few homology to the other proteins by molecular cloning and sequence analysis. In this study, we investigated the physiological role of SspA under various stress conditions. Using recombinant SspA expressed in *E. coli* as an antigen, polyclonal antibody for SspA was prepared. First, using the SspA antibodies, we identified localization of SspA as outer membrane by using sucrose density gradient centrifugation and electron microscopy. Second, expression of SspA was analyzed in the following various growth conditions: salt stresses (0.68 M NaCl or KCl), osmotic stress (0.68 M sorbitol), acid stress (pH 6.0), cold stress (0°C for 2 h), and heat stress (42°C for 2 h). Western blot analysis demonstrated that SspA was highly induced by salt stresses in both anaerobic and aerobic conditions compared to other conditions. Third, we constructed *sspA*-disrupted mutant, and analyzed its phenotype with wild type strain. While *sspA*-disrupted *R. sphaeroides* grew normally in the anaerobic photosynthetic condition in the presence or absence of stresses, it displayed significantly retarded growth in the aerobic dark condition, especially when osmotic or salt stresses were imposed. *sspA* disruptant, but not the wild type, formed cell aggregates when grown in both aerobic and aerobic growth conditions, which was significantly enhanced in the salt-stressed aerobic growth condition. In conclusion, SspA may play an important role in the salt-stressed aerobic growth conditions.

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UNDERSTANDING THE SALT RESPONSE OF A HALOTOLERANT CYANOBACTERIUM *EUHALOTHECE* SP. BAA001 USING PHYSIOLOGICAL AND PROTEOMIC ANALYSIS

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Euhalothece sp. BAA001, an extremely halotolerant cyanobacterium isolated (at a salinity of 88 g/l) from a Libyan hypersaline lake (Qabar-Onn), was subjected to physiological and proteomic analysis. *Euhalothece* sp. BAA001 was identified based on microscopy (ultrastructure using SEM and TEM) and 16S rRNA gene sequence analysis (GenBank Accession Number AY457568). Based on the 16S rDNA distance tree *Synechocystis* sp. PCC 6803 is a near relative. After identification, *Euhalothece* sp. BAA001 was cultured at 28 ºC with 12 hrs light/dark cycles during the incubation period in BG-11 medium with 1.6, 40, 60, 88 and 120 g/l of added NaCl at pH ranging from 4.5 to 10.5. The growth rate (in triplicate) was measured based on optical density (OD) absorbance at 530 nm and Chlorophyll-a concentration. At pH 7 and 9, all cultures showed sustained growth that was highly dependent on the salinity of each culture. No growth was observed for pH of 4.5 and 10. The experiments showed that maximum growth occurred in cultures containing 60 g/l NaCl for both pH 7 and 9. This was accompanied by large variations in pigmentation of the cultures, with cultures greener for longer at lower concentrations of NaCl, and yellower at higher salinity. Subsequently, the global protein variations were analysed using large format 2-D electrophoresis, in triplicate, with both broad range (pH 3-10) and narrower range (pH 4-7) IEF strips. Over 250 spots were generated per phenotype, with changes assessed using PDQuest. Analysis of trypsin-generated peptide tags from differentially expressed proteins was undertaken using an electrospray ionization quadrupole time of flight mass spectrometer (QStar XL, Applied Biosystems) equipped with a capillary liquid chromatograph (LC Packings). Peptide mass fingerprints from MS and MS-MS studies were analysed using Pro-ID against the whole non-redundant protein database at NCBI and the theoretical proteome of *Synechocystis sp.* PCC 6803. A number of proteins were related to photosynthetic apparatus-like pigments, such as phycocyanin and allophycocanin, whilst the majority of the common proteins are involved in the respiratory electron transport pathway. This work shows the utility of proteomics in characterising the physiology of an environmental cyanobacterial isolate.

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GENETIC EVIDENCE IDENTIFYING THE TRUE GLUCONEOGENIC FRUCTOSE-1,6-BISPHOSPHATASE IN *THERMOCOCCUS KODAKARAENSIS* AND OTHER HYPERTHERMOPHILES

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Fructose-1,6-bisphosphatase (FBPase) is one of the key enzymes in gluconeogenesis. Although FBPase activity has been detected in several hyperthermophiles, no orthologs corresponding to the classical FBPases from bacteria and eukaryotes have been identified on their genomes. An inositol monophosphatase (IMPase) from *Methanococcus jannaschii* which displayed both FBPase and IMPase activities, and a structurally novel FBPase (*Tk*-Fbp) from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1, have been proposed as the "missing" FBPase. Here, using *T. kodakaraensis*, we took a genetic approach to elucidate which candidate is the major gluconeogenic enzyme *in vivo*. The IMPase/FBPase ortholog in *T. kodakaraensis*, *Tk*-Imp, was confirmed to possess high FBPase activity along with IMPase activity as in the case of other orthologs. We therefore constructed Ä*fbp* and Ä*imp* strains by applying the gene disruption system recently developed for *T. kodakaraensis*, and investigated their phenotypes. The Ä*fbp* strain could not grow under gluconeogenic conditions while glycolytic growth was unimpaired, and the disruption resulted in the complete abolishment of intracellular FBPase activity. Evidently, *fbp* is an indispensable gene for gluconeogenesis and is responsible for almost all intracellular FBPase activity. In contrast, the endogenous *imp* could not complement the defect of *fbp*, and its disruption did not lead to any detectable phenotypic changes under the conditions examined. These facts indicated that *imp* is irrelevant to gluconeogenesis despite the high FBPase activity of its protein product, probably due to insufficient transcription. Our results provide strong evidence that the true FBPase for gluconeogenesis in the hyperthermophile is the *Tk*-Fbp ortholog, and not the IMPase/FBPase ortholog.

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MANNOSYLGLUCOSYLGLYCERATE, A NOVEL SOLUTE OF THE THERMOPHILIC BACTERIUM *PETROTOGA MIOTHERMA*

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Petrotoga miotherma is a thermophilic bacterium with a sheath-like outer structure ("toga"), which was first isolated from petroleum oil reservoirs in Oklahoma and Texas. This bacterium is obligatory anaerobic, fermentative, and capable of reducing elemental sulfur to hydrogen sulfide. It can grow in the temperature range from 35 to 65ºC, and with salt concentrations from 0.5% to 10% NaCl [1]. A large variety of microorganisms, ranging from archaea, bacteria, yeast, filamentous fungi and algae rely exclusively on the accumulation of compatible solutes for osmoadaptation. Our group has

examined many thermophilic and hyperthermophilic microorganisms isolated from marine environments for the presence of low molecular mass solutes and has characterised several novel compatible solutes [2]. Thermophiles and hyperthermophiles accumulate compatible solutes that have not been found, or have been rarely encountered in mesophilic organisms leading to the view that the compatible solutes of (hyper)thermophiles are specifically associated with life at high temperatures. These compatible solutes are generally negatively charged, as are many of those of archaea, while mesophiles generally accumulate neutral or zwitterionic compatible solutes. Proton NMR of ethanolic extracts of *P. miotherma* led to the identification of a new sugar derivative accumulating in this organism. The molecular structure of the unknown compound is á (1-2) mannopyranosyl á (1-2) glucopyranosyl-glycerate. It is a derivative of glucosylglycerate, a compatible solute found in a few microorganisms. In addition to mannosylglucosylglycerate several osmoactive amino acids like proline, alanine and glutamate were identified. The effect of the growth temperature, salinity and oxidative stress conditions on the accumulation of intracellular organic solutes by *P. miotherma* was also investigated. At the optimum growth temperature (55ºC) and optimum NaCl concentration in the medium (3%), mannosylglucosylglycerate was the major solute accumulated (0.6 ìmol/mg protein) by *P. miotherma*. The levels of this solute decreased progressively as the growth temperature or the salinity of the medium was raised above the optimum. The ability of mannosylglucosylglycerate to protect malate dehydrogenase (MDH) against heat or dissecation was also evaluated. In the absence of solutes the activity of MDH was nearly completely lost at 45ºC. However, in the presence of mannosylglucosylglycerate, 38% of MDH activity was retained at 50ºC and 26% at 53ºC. Interestingly, mannosylglycerate, a closely related solute, showed a much higher efficacy.

[1] Davey, M. E., Wood, W. A. Key, R., Nakamura, K., Stahl, D. A. *System. Appl. Microbiol.* **16**, 191-200 (1993)
[2] da Costa, M., Santos, H; Galinski, E. A. *Adv. Biochem. Eng. Biotech.* **61**, 117-153 (1998)

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ENRICHMENT AND ISOLATION OF THERMOPHILIC ORGANISMS CAPABLE OF FERMENTING ORGANIC POLYMERS

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Thermophilic and hyperthermophilic organisms capable of fermenting organic polymers were enriched from production water from a North Sea oil well with various organic polymers as substrates. Growth was observed in enrichments with á-cellulose, avicel, xylan, chitin or peptone as substrates and energy sources. Based on 16S rDNA analysis, isolates from these enrichments were identified as members of the *Thermotogales* (á-cellulose enrichment) and *Thermococcus* (peptone enrichment) genera. Isolates from the chitin enrichment seem to belong to a new genus related to the *Aminobacterium* and *Dethiosulfovibrio* genera. Extensive characterisation of the isolated *Thermococcus* strain has been performed. This strain is an obligately anaerobic heterotroph able to utilize yeast extract and peptone. Growth is stimulated by sulfur. Temperature and pH optima are 83°C and 6,7 respectively. One of its closest relatives is *Thermococcus sibirucus* wich is, so far, the only *Thermococcus* species reported to be isolated from an oil reservoir.The isolated *Thermotogales* strain TC12, has *Thermotoga maritima* as its closest relative (99% 16S rDNA homology). Strain TC12 can however be differentiated from *T. maritima* by a lower temperature optimum (55-60°C compared to 80°C for *T. maritima*).

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CHARACTERIZATION OF THIOREDOXIN REDUCTASE ACTIVE WITH NADH OR NADPH PURIFIED FROM *DEINOCOCCUS RADIOPHILUS*

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The Genus *Deinococcus*, an obligate aerobic, non-sporeforming bacterium, has many peculiar features of thick cell wall-contained L-ornithine, an outer memrane-like structure, and a membrane-bound carotenoid pigment The major peculiarity of *Deinococcus* is its extraordinarily resistance against UV, ionizing radiations, and oxidative stress. For understanding its resistant physiology, reactive oxygen species (ROS) scavenging enzymes of *Deinococcus* spp. are worthwhile to be investigated. Thioredoxin reductase (TrxR, EC 1.6.4.5) belonging to the flavoprotein family of pyridine nucleotide-disulfide oxidoreductase is a member of ROS scavenging emzymes occurring ubiquitously in Archaea, Bacteria, and Eukarya and is indispensible for an intracellular redox homeostasis and protection cells from oxidant toxicity. *Deinococcus radiophilus* seems to own isoforms of TrxR, one active with NADH or NADPH (a major abundant form) and the other is specific for NADPH. The TrxR showing dual specificity was purified to homogeneity from the cell-free sonic extract by steps of ammonium sulfate fractionation, 2'5'ADP-Sepharose 4B affinity chromatography, and Sephadex G-100 gel filtration. The purified TrxR gave a 368 U/mg protein of specific activity (with NADPH) with 478-fold purification and 18% recovery from the cell-free extract. The molecular weight of the purified TrxR estimated by native-PAGE and gel filtration is 63.1 and 72.2 kDa, respectively. The subunit molecular weight of TrxR is 37 kDa. An isoelectric point of the TrxR is ca. 4.5. The presence of FAD in the purified TrxR was confirmed by absorbance at 460 nm. With 5'5-dithio-bis-2-nitrobenzoic acid (DTNB), the substituted substrate for thioredoxin, the Km and Vmax are 463 ìM and 756 ìM/min, respectively. The Km and Vmax of TrxR for NADPH are 12.5 ìM and 25 ìM/min, respectively, whereas those for NADH are 30.2 ìM and 192 ìM/min, respectively. The pressnce of FAD in TrxR was confirmed by absorbance at 460 nm. The purified TrxR was quite stable in range of pH 3 to 9, and relatively thermostable upto 70 °C, however, the 50% reduction of its activity occurred at 80 °C. A drastic inhibition of TrxR activity occurred by Cu^{2+}, Zn^{2+}, Hg^{2+}, and Cd^{2+}, but a moderate reduction (ca. 50-60%) by Ag^{1+}. A significant inhibition of TrxR by N-ethylmaleimide suggests an important role of cysteine in its activity. N-terminal amino acid sequences of the purified *D. radiophilus* TrxR (Ser-Glu-Gln-Ala-Gln-Met-Tyr-Asp-Val-Ile-Ile-Val-Gly-Gly-Gly-Pro-Ala-Gly-Leu-Tyr-Ala-) show high similarity with those of TrxRs reported in *Archaea* such as*, Methanosarcina mazei, Archaeoglobus fulgidus,etc.*

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DYNAMICS OF THE METABOLITE POOL IN METHANOCOCCUS JANNASCHII

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NMR techniques have been used to monitor incorporation of ^{13}C-formate into the small molecule pool of *Methanococcus jannaschii* as a function of both temperature and osmotic stress. The major small molecules in this hyperthermophile are á- and â-glutamate, although many other solutes occurring at low levels can be identified. Turnover of the glutamate pools can be measured by 103 m°P;ï•□ chasing m°P;ï•□ the ^{13}C label supplied by formate with a 10-fold excess of unenriched sodium formate. In control cells, the sudden increase in unenriched formate causes an increase in the ^{13}C content of á-glutamate synthesis (followed by loss of ^{13}C on a longer time-scale) which precedes an increase in ^{13}C content of â-glutamate by about 5 min. An increase in external NaCl caused an increase in both á- and â-glutamate within 10-30 min; ^{13}C content increased initially indicating the synthesis of these solutes from pre-exisiting labeled precursors following by loss of ^{13}C label as the solutes were used for cell growth (protein synthesis in the case of á-glutamate). Interestingly, ^{13}C in the â-glutamate pool also was chased into an NMR-invisible pool indictaing that in these cells (in contrast to other methanogens that accumulate this solute), the molecule can be converted to other species. More detailed analyses of *M. jannaschii* extracts labeled with ^{13}C precursors (^{13}C-formate or $^{13}CO_2$) provide information on how the metabolome changes under these stress conditions. The identification and quantitation of these solutes will also be discussed.

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THERMOSIPHO ATLANTICUS SP. NOV., A NOVEL MEMBER OF *THERMOTOGALES* ISOLATED FROM A MID-ATLANTIC RIDGE HYDROTHERMAL VENT

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A novel anaerobic, thermophilic and heterotrophic bacterium, designated DV1140^T, was isolated from a deep-sea hydrothermal vent sample from the Mid-Atlantic Ridge. The cells were non motile straight rods of 1.8 µm long and 0.4 µm wide, surrounded by an outer sheath-like structure (toga). They grew between 45 °C and 80 °C (opt. 65 °C), between pH 5.0 and 9.0 (opt. pH 6.0) and between 20 and 60 g sea salts l^{-1} (opt. sea salts concentration 30 g l^{-1}). Strain DV1140^T was able to ferment yeast extract, peptone, brain-heart infusion, gelatin, starch, cellobiose, trehalose, arabinose, glucose and galactose. The fermentation products identified on glucose in presence of yeast extract and peptone were acetate, isovalerate and hydrogen. Strain DV1140^T is able to grow at O_2 concentrations up to 8% in the presence of elemental sulfur and up to 4% without elemental sulfur. Its growth is inhibited with 29 % H_2 in the gas phase. The requirement for an external electron acceptor was tested. Only a slight enhancement of growth yield was observed in the presence of cystine. No difference on growth kinetics and no significant maximum cell concentrations were noticed during cultures with or without elemental sulfur. Polysulfides, sodium thiosulfate, sodium sulfite, sodium sulfate, sodium nitrite and sodium nitrate did not enhance growth. The G+C content of the genomic DNA was 33 mol %. Phylogenetic analysis of the 16S rRNA gene (GenBank accession number AJ577471) located the strain within the genus *Thermosipho* in the bacterial domain. On the basis of 16S rDNA sequence comparisons, physiological and biochemical characteristics, we propose that the isolate should be described as a novel species: *Thermosipho atlanticus* sp. nov.. The type strain is DV1140^T (=CIP 108053^T, =DSM 15807^T).

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THERMOTOLERANCE OF THERMOCOCCUS SP. STRAIN TC-1-95 AT ULTRA HIGH TEMPERATURE

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Thermotolerance of hyperthermophiles is basic information for estimating the ecological distribution of hyperthermophiles in submarine and terrestrial hydrothermal systems, as well as for understanding the mechanism of their thermal adaptation. We have developed a new flow-type apparatus in order to study thermotolerance of hyperthermophiles under conditions simulating submarine hydrothermal circulation. The apparatus is designed so that a cell culture is heated at high temperatures only for a few seconds. It can be operated at temperatures up to 400 °C and hydrostatic pressures up to 30 MPa either aerobically or anaerobically. Using this apparatus, we investigated the thermotolerance of Thermococcus sp. strain Tc-1-95, which was isolated at a hydrothermal vent in the Central Indian Ridge (Takai et al., 2004) and grows between <80 and 102 °C (optimum 90 °C). The cell culture was heated at temperatures between 95.0 and 125.0 °C for 1.5 sec, and the survival rate was measured. Experiments were performed at 0.5 MPa and 25 MPa, the latter of which corresponds to the hydrostatic pressure at the depth of isolation. At 0.5 MPa, no decrease of the survival rate was observed even at 114 °C. The survival rates decreased with temperature above 114 °C, and no viable cell was detected at 122 °C. The thermostable temperature was raised up to 118 °C, when the hydrostatic pressure of 25 MPa was applied. By batch-wise experiments with heating time for some hours, it has been reported that thermotolerance of Pyrococcus sp. strain ES4 and Pyrococcus abyssi are facilitated by about 3 °C when hydrostatic pressure of 20 MPa is applied (Holden and Baross, 1995; Marteinsson et al., 1997). Our result indicates that similar adaptation mechanism comes into effect even when the heating time is considerably short. By microscopic observations, it was suggested that the thermal death of Thermococcus sp. strain Tc-1-95 is induced by rupture of the cell at high temperatures. This is in

marked contrast to the thermal death of E. coli, where thermal denaturation of ribosome may be the primary process (Mackey et al., 1991) and is not associated with morphological change of the cell.

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SPECIAL FEATURES OF THE ATP SYNTHASE ARE REQUIRED FOR OXIDATIVE PHOSPHORYLATION BY ALKALIPHILIC *BACILLUS* AT HIGH PH

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Oxidative phosphorylation (OXPHOS) by extremely alkaliphilic *Bacillus* species represents a major energetic conundrum with respect to the dominant model for energy-coupling in OXPHOS. The Mitchell chemiosmotic model posits a direct relationship between the magnitude of a bulk electrochemical gradient of protons and the capacity for ATP synthesis and also posits that an artificially imposed electrochemical gradient should be as efficacious as one generated by the respiratory chain. OXPHOS by alkaliphilic *Bacillus pseudofirmus* OF4 runs counter to both of these tenets. This organism synthesizes ATP via OXPHOS more robustly at pH 10.5 than at pH 7.5, although the total bulk chemiosmotic driving force is greatly reduced at the higher pH. In addition, an imposed diffusion potential loses its efficacy in energizing ATP synthesis above about pH 9.2 while OXPHOS itself is robust well above that pH (i.e. when respiration itself is generating the driving force). We have hypothesized that special features of the membrane-embedded subunits of the ATP synthase constitute: (i) a pH-dependent gating function that prevents proton loss to the bulk during OXPHOS at high pH and also accounts for the inability of imposed potentials to energize above pH 9.2; and (ii) a mechanism for acquiring protons above the gating pH of 9.2 in a manner that is sequestered from the highly alkaline bulk phase. Six sequence-specific features of the *a*- and *c*-subunits of the *B. pseudofirmus* OF4 ATP synthase have now been changed to the non-alkaliphilic *Bacillus* consensus sequence. All but one of these changes allowed assembly of a functional ATP synthase, as assayed at pH 7.5. The results of a detailed bioenergetic profile strongly support specific roles for the remaining features in OXPHOS at pH 10.5 but not at pH 7.5. Support for a role for three of these features in proton gating at high pH also emerged. Thus adaptations of the OXPHOS machinery itself are required for alkaliphile OXPHOS at high pH in addition to possible global mechanisms for proton retention near the membrane surface.

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A NOVEL COENZYME F420-DEPENDENT SULFITE REDUCTASE IN THE METHANOGENIC ARCHAEA

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Methanocaldococcus jannaschii, a strictly anaerobic chemolithoautotrophic hyperthermophilic methanogenic archaeon lives within submarine hydrothermal vents and receives nutrients from the vent fluid. A mixing with aerobic cold seawater permeating through the vent wall brings the temperature of the sulfide-containing vent fluid down from 350 oC to temperatures at which M. jannaschii can grow (48-94 oC). The reaction of sulfide with oxygen helps to maintain anaerobic conditions, but it has the potential of producing sulfite. Sulfite is a strong inhibitor of methanogenesis. We show that M. jannaschii grew with sulfite as the sole sulfur source and during this process it expressed a polypeptide at a high level. We identified this polypeptide as MJ0870, an ORF annotated in the NCBI database as b-subunit of a coenzyme F420-reducing hydrogenase. We found the NH2-terminal half of MJ0870 carried a H2F420-dehydrogenase domain and the COO—terminal half was similar to the A subunits of sirohaeme-containing dissimilatory sulfite reductases. We call MJ0870 a F420-dependent sulfite reductase (FSR). FSR homologs were found in Methanothermobacter thermautotrophicus and Methanopyrus kandleri. Extracts of M. jannaschii cells grown with sulfite, but not with sulfide, oxidized H2F420 with sulfite, and this activity was seen also with nitrite but not with nitrate or sulfate. The FSR activity was highly oxygen sensitive and associated with the membrane and heavier soluble fraction of cell extracts. We concluded that M. jannaschii uses a novel coenzyme F420-dependent sulfite reductase (FSR) for generating sulfide from sulfite and a high enzyme level and an association with the membrane helps the cell to avoid the toxicity of sulfite. Prior to our work the H2F420 dehydrogenase function was thought to be absent in strictly hydrogenotrophic methanogen such as M. jannaschii. In methylotrophic methanogens such as the Methanosarcina species H2F420 dehydrogenase is a part of the membrane-resident electron transport chain. Thus, it is possible that this electron shuttling protein was recruited for methylotrohic methanogenesis from a sulfite reductase of the deeply rooted archaea and we present supporting evolutionary deductions in this line. Evolution of sulfite reduction is also discussed.

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EXCEPTIONS OF THE RULE: EVIDENCE FOR DIMERIC ACONITASES IN *SULFOLOBUS SP.*

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Aconitase belong to a class of dehydratases, which employ a redox-inactive but oxygen labile iron sulfur cluster for catalysis [1]. Besides their role as enzymes of the central carbon metabolism, the clusterfree apoforms of some bifunctional aconitases also act as

post-transcriptional regulators, involved in iron homeostasis and oxidative stress response [2]. Little is known about these interesting proteins from *Archaea*. Recently, we have published the characterization of the first archaeal aconitase from the hyperthermophilic *Sulfolobus acidocaldarius* and established its function in both the citric acid and the glyoxylate cycle [3,4]. Furthermore, we succeeded in overexpression of the enzyme from *S. solfataricus* in *E. coli* [5]. The co-expression of a cysteine desulfurase from a cyanobacterium resulted in higher amounts of catalytically active recombinant aconitase. Increased cluster insertion in co-expressing cells was directly confirmed by EPR measurements. The crenarchaeal aconitase was purified to a specific activity of about 150 U/mg and to more than 95% homogeneneity by a semianaerobic procedure. In striking contrast to known aconitases from *Eucarya* or *Bacteria* (all described as monomers), both *Sulfolobus* aconitases are homodimers. Surprisingly, dimerization depends on the presence of the iron sulfur cluster. This indicates an important structural role for the metal cofactor. So far, this feature is unique to the *Sulfolobus* enzymes, as aconitases from other thermophilic *Archaea* and *Bacteria* exist only in the typical monomeric state. *In silico* analysis revealed two highly charged regions in the crenarchaeal enzymes, which might serve as dimer interfaces. Interestingly, in iron starved cells, a small part of the *S. acidocaldarius* aconitase was found in the monomeric state, i.e. clusterfree apo form, which is the prerequisite of RNA binding capacities of known bifunctional aconitases. [**1**] Beinert H. et al., 1996. Chem. Rev. 96: 2335-74; [**2**] Eisenstein R.S. 2000. Annu. Rev. Nutr. 20: 627-62; [**3**] Uhrigshardt H. et al., 2001. Eur. J. Biochem. 268(6): 1760-71; [**4**] Uhrigshardt H. et al., 2002a. FEBS Lett. 513 (2-3): 223-29; [**5**] Uhrigshardt H. et al., 2002b. Biochem. Soc. Trans. 30(4): 685-87.

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EVIDENCE FOR AUTOTROPHIC CO2 FIXATION VIA THE REDUCTIVE TRICARBOXYLIC ACID CYCLE IN MEMBERS OF THE EPSILON-PROTEOBACTERIA

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Today, representatives that are able to grow autotrophically exist in almost all major groups of prokaryotes. These organisms play essential roles in ecosystems by providing a continuous supply of organic carbon for heterotrophs. At present there are four pathways known, the Calvin cycle, the reductive tricarboxylic acid cycle (rTCA), the reductive acetyl-CoA pathway, and the hydroxypropionate pathway. Members of the epsilon-proteobacteria appear to be a major component of microbial communities at deep-sea vents and many are chemolithoautotrophs. However, no information exist what carbon fixation pathway is used by epsilon-proteobacteria. We present biochemical and genetic evidence for the operation of the rTCA cycle in two members of the Îµ-proteobacteria, Candidatus Arcobacter sulfidicus and Thiomicrospira denitrificans. We have measured the activities of all enzymes of this cycle by using diagnostic enzyme assays, including the ATP citrate lyase (ACL), 2-oxoglutarate:acceptor oxidoreductase, and pyruvate:acceptor oxidoreductase. We also amplified the genes coding for ACL. Recently genes coding for ACL have been identified on fosmids of the main epsilon-proteobacterial epibiont of Alvinella pompejana, a polychaete living at deep-sea vents. Together, this indicates that the rTCA cycle might be a common pathway used by epsilon-proteobacteria for carbon fixation, significantly contributing to biomass production at vents. The finding of the rTCA cycle in epsilon-proteobacteria has also evolutionary significance. Together with delta-proteobacteria, epsilon-proteobacteria are believed to represent the most ancient subdivision of the proteobacteria and they might have shared a common ancestor with a group containing green sulfur bacteria and Aquificales, several species of which use the rTCA cycle.

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CHARACTERIZATION OF THERMOPHILIC, CHEMOLITHOAUTOTROPHIC NITRATE-REDUCING BACTERIA FROM DEEP-SEA HYDROTHERMAL VENTS, AND IDENTIFICATION OF THE GENES INVOLVED IN NITRATE REDUCTION AND CARBON FIXATION

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Recent studies suggest that novel thermophilic microorganisms that couple the reduction of nitrate with autotrophic CO2 fixation may play an important role in both the nitrogen cycling and primary production in marine geothermal environments. We obtained several thermophilic, nitrate-reducing isolates originating from active sulfide samples collected on both the East Pacific Rise and the Mid-Atlantic Ridge. These isolates include the newly described species Thermovibrio ammonificans (Topt: 75°C; phylum Aquificae), and a novel species related to the epsilon-proteobacteria (Caminibacter strains TB-1 and TB-2; Topt: 55°C). These isolates are strictly anaerobic, chemolithoautotrophic bacteria, and reduce nitrate to ammonium, using hydrogen as the electron donor. Alternatively they can grow by reduction of sulfur to hydrogen sulfide. Recently, we cloned and sequenced the gene encoding for a NapA-type nitrate reductase from four Caminibacter strains (including TB-1 and TB-2). This is the first identification of a napA gene in thermophilic bacteria, and our finding is consistent with the presence of this gene in mesophilic members of the epsilon proteobacteria, such as Sulfurospirillum barnesii and Wolinella succinogenes. We also cloned and sequenced the gene encoding for the enzyme ATP citrate lyase from T. ammonificans, and we have evidence for the presence of this gene in the Caminibacter strains TB-1 and TB-2. These findings strongly suggest that the reductive TCA cycle for autotrophic carbon fixation might be operating in both T. ammonificans and Caminibacter ssp. The ecological significance of chemolithotrophic, nitrate-reducing organisms at deep-sea hydrothermal vents is twofold: 1) they contribute to the primary productivity by fixing CO2, and 2) their nitrate respiratory metabolism (namely, the reduction of NO3- to NH4+) implicate that nitrogen is conserved within the vent microbial community and thus conserved within the vent system.

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A NOVEL PROTEIN SECRETION PATHWAY IN THE ANTARCTIC BACTERIUM PSEUDOALTEROMONAS HALOPLANKTIS TAC125: THE PSYCHROPHILIC ANSWER TO A KEY QUESTION

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Extra-cellular protein secretion is a central aspect in bacterial physiology, being the means, among others, to interact and modify the surrounding environment. This is a crucial aspect for microorganisms able to survive in hostile extreme habitats, particularly, in the cold. Although several psychrophilic exo-enzymes have been characterized, nothing is virtually known about the mechanisms responsible for their extra-cellular addressing. The development of a cold-adapted shuttle vector [1] allowed us to investigate on the cellular machineries devoted to protein secretion in Antarctic bacterial hosts. The secretion of the psychrophilic á-amylase from Pseudoalteromonas haloplanktis TAB23 [2] was chosen as case study. Previous results demonstrated that the á-amylase is an odd exo-enzyme making use of two alternative secretion pathways depending on the bacterial host which is expressed in, i.e. a propeptide-mediated â-autotransporter-like mechanism in E. coli and a Type II-like route in three Antarctic bacteria (PhTAB23, the parent strain, PhTAC125, and Psychrobacter sp. TAD1) [3]. An in vivo complementation experiment was set up to identify the genes responsible for the á-amylase secretion in PhTAC125. By applying this strategy, a 37.5 Kb long genomic fragment was selected, whose nucleotide sequence was determined and in silico analyzed. Surprisingly, the selected DNA portion does not code for neither a Type II secretion apparatus components nor for any of the already characterised secretion pathways [4]. These results are strongly suggestive of a novel secretion machinery occurring in the Antarctic PhTAC125 strain. An extensive mutagenesis analysis to identify the psychrophilic functions specifically involved in the extra-cellular targeting is in progress. Our research group is presently involved in an European consortium aimed at sequencing and annotating PhTAC125 genome. This work represents a contribution toward the final goal of unraveling the molecular adaptations evolved by cold-adapted bacteria to survive in the extreme Antarctic environment.

1. Tutino M. L., et al. 2001. Extremophiles. 5:257-264.
2. Feller G., et al. 1992 J Biol Chem. 267:5217-5221.
3. Tutino M. L., et al. 2002. J Bacteriol. 184:5814-7.
4. Lee V.T. and Schneewind O. 2001. Gen Develop. 15:1725-1752.

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METABOLIC AND PHYLOGENETIC DIVERSITY OF THERMOPHILIC HYDROGENOGENIC CO-OXIDIZING PROKARYOTES

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Distribution, phylogeny and metabolic diversity of hydrogenogenic CO oxidizing anaerobes were studied. Two new bacterial genera *Thermoincola* gen. nov. and *Thermosinus* gen. nov. were isolated from terrestrial hot springs. Both organisms were shown to be moderate thermophiles and obligate anaerobes oxidizing CO and producing equimolar quantities of H_2 and CO_2. *Thermoincola carboxydophila* sp. nov. was shown to be obligate chemolithotroph and, as the previously known microorganisms of this group, did not reduce any electron acceptors. *Thermosinus carboxydivorans* sp. nov. was shown to be facultative carboxydotroph capable of organotrophic growth. During the growth on CO it was able to reduce Fe(III) to Fe(II) and selenite to elemental selenium, but could grow without electron acceptors. Contrary to it, a new hydrogenogenic carboxydotrophic isolate obtained from Kamchatka turned to be obligately dependent on the reduction of ferric iron. These data show that the process of anaerobic CO oxidation by hydrogenogenic thermophilic prokaryotes can be coupled with the reduction of inorganic electron acceptors. From hydrothermal venting structures collected at East Pacific Rise 13°N a hyperthermophilic archaeon capable of lithotrophic growth on CO coupled with equimolar production of molecular hydrogen was isolated. The growth of the new isolate on CO was stimulated by thiosulfate which was reduced to H_2S. Based on its 16S rRNA sequence analysis, this organism was affiliated to the genus *Thermococcus*. This is the first evidence of anaerobic CO oxidation coupled with H_2 production performed by hyperthermophilic Archaea.

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INVOLVEMENT OF REVERSE GYRASE IN UV RESPONSE IN SULFOLOBUS SOLFATARICUS

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Induction of DNA damage triggers a complex biological response concerning not only repair systems, but also virtually every cell function. DNA topoisomerases regulate the level of DNA supercoiling in all DNA transactions. Reverse gyrase is a peculiar DNA topoisomerase, specific of hyperthermophilic microorganisms, containing a helicase and a topoisomerase IA domain, which has the unique ability of introducing positive supercoiling into DNA molecules. We show here that reverse gyrase of the archaeon Sulfolobus solfataricus is mobilized to DNA in vivo after UV irradiation. The enzyme, either purified or in cell extracts, forms stable covalent complexes with UV-damaged DNA in vitro. We also show that the reverse gyrase translocation to DNA in vivo and

stabilization of covalent complexes in vitro are specific effects of UV irradiation and do not occur with the intercalating agent actinomycin D. Our results suggest that reverse gyrase might participate, directly or indirectly, in the cell response to UV-induced DNA damage. This is the first direct evidence of recruitment of a topoisomerase IA enzyme to DNA after induction of DNA damage. The interaction between helicase and topoisomerase activities has been previously proposed to facilitate aspects of DNA replication or recombination in both Bacteria and eukaryotes. Our results suggest a general role of the association of such activities in maintaining genome integrity and a mutual effect of DNA topology and repair.

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WHOLE GENOME TRANSCRIPTIONAL ANALYSIS OF ACETATE-INDUCED DYNAMIC STRESS RESPONSE IN THE HYPERTHERMOPHILIC BACTERIUM THERMOTOGA MARITIMA

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The hyperthermophilic bacterium Thermotoga maritima produces short chain fatty acids, primarily acetate, as fermentative products during growth at 80°C. This leads to a significant reduction in the culture pH, which can ultimately limit growth if the medium is not buffered or if pH control is not used. While a number of studies have looked at the acetate response of mesophilic and moderately thermophilic bacteria, this issue has not been examined to any extent in fermentative hyperthermophiles. Under normal growth conditions, mesophilic microorganisms are known to actively transport acetate out of the cytoplasm to prevent its toxic buildup within the cell. However, a toxicity issue arises with the increasing concentration of both extra cellular and intracellular acetate. This deleterious action is compounded at lower pH, due to more favorable conditions for acetate membrane diffusion and proton liberation. While acetate has been shown to inhibit several cellular processes, the most prominent effect is a decrease in the transmembrane proton gradient. Several studies with Escherichia coli focusing on the global effects of weak acids have demonstrated a complex response involving general stress proteins, metabolic proteins, and transcriptional regulators. To examine this issue for T. maritima, a 14-liter high temperature fermenter was used to follow the transcriptional response to a step increase in external acetate concentration. Of particular interest was the role of proteolysis in the acid stress response, in light of the importance of this aspect of cellular metabolism in less thermophilic microorganisms. Results will be reported that show gene expression patterns to acetate challenge in T. maritima as these relate to similar phenomena in mesophilic and moderately thermophilic bacteria.

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GENETIC ANALYSIS OF TREHALOSE AND MANNOSYLGLYCERATE PRODUCING ENZYMES IN *THERMUS* SPP. AND THEIR RELEVANCE FOR OSMOTOLERANCE

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The organisms of the genus *Thermus* are thermophilic (T_{op}H"70°C) with different degrees of osmotolerance, ranging from 1 to 5% NaCl. Recently, the *otsA* and *otsB* genes from *T. thermophilus* RQ-1 were cloned and sequenced. These genes are immediately followed by *treS*, and are structurally linked. The importance of *otsA* and *otsB* genes for osmoadaptation was proven since partial deletion of these genes greatly affects salt tolerance of *T. thermophilus* RQ-1 (1). Interestingly, the screening for the presence of trehalose synthesizing genes in other *T. thermophilus* strains revealed different genetic organization among this group. In strains T-2, PRQ-14, Fiji 3A1, CC-16 and B trehalose genes showed the same organization as in RQ-1, whereas strains GK24, HB8, AT-62 lack *otsA* and strain HB27 lack the 3 genes (*otsA*, *otsB* and *treS*). The genes responsible for the synthesis of trehalose were also absent in organisms belonging to *T. aquaticus, T. scotoductus*, *T. antranikianii*, *T. filiformis, T. igniterrae*, *T. brockianus* and *T. oshimai*. The presence of mannosylglycerate synthesizing genes (*mpgs* and *mpgp*) was also screened, revealing that these genes were exclusive of *T. thermophilus* strains. However, strain CC-16 belonging to *T. thermophilus* did not possess these genes. In the present work, we have used a growth medium deprived of external trehalose to assess the salt tolerance limits of the organisms and to establish a correlation between the presence of the genes for the synthesis of the compatible solutes trehalose and mannosylglycerate and osmotolerance.

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BIOSYNTHESIS OF MANNOSYLGLYCERATE IN CRENARCHAEOTA

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In the present study we identified two genes from the hyperthermophilic crenarchaeon *Aeropurum pernix* coding for enzymes responsible for the synthesis of mannosylglycerate (MG). MG is a common compatible solute of thermophilic and hyperthermophilic halotolerant prokaryotes (Santos and da Costa, 2002). Blast searches with *A. pernix* genome revealed an ORF with homology to mannosyl-3-phosphoglycerate synthase (MPGS) and to mannosyl-3-phosphoglycerate phosphatase (MPGP) from thermophilic and hyperthermophilic bacteria and archaea (Empadinhas et al., 2001; Empadinhas et al., 2003). These enzymes catalyze two consecutive steps in the biosynthesis of MG. The overexpression of *mpgs* in *Escherichia coli* was hampered in codon usage and context, as

determined by Anaconda 1.0 software analysis. For this reason we optimized the nucleotide sequence of *mpgs* for *E. coli* expression and synthesized the gene using a megaprimer approach. Recent results showed the presence of an *mpgs*-homologue in uncultured mesophilic archaea (Schlepper et al., 1998). We overexpressed the *mpgs*-homologue in *E. coli*. Initially, the *mpgs*-overexpressing clone did not synthesize MPG although a huge expression band was visible on SDS-PAGE. We, therefore, used a strain containing tRNA genes for the codons rarely used by *E. coli*, for expression of mpgs. Incubation of the cell-free extracts at 30ºC with GDP-mannose and 3-PGA revealed MPG synthesis, validating the genuine function of this gene. We cloned this gene in vectors that produce His-tagged proteins to assist purification. The presence of genes for synthesis of MG in mesophilic archaea is of great physiological and evolutionary novelty. Notwithstanding the fact that the role of MG in these uncultured organisms is elusive and calls for further investigation, the data presented here appear to do not support the idea that MG tracks thermophily.

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PRESSURE PROBES THE DYNAMICS OF MEMBRANE PROTEINS IN SACCHAROMYCES CEREVISIAE

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There has been a renewal of interest in the investigation of the survival strategies employed by deep-sea piezophiles as well as the effects of high hydrostatic pressure in mesophiles. Studies with mesophiles have demonstrated that high pressure inhibits cell division, nutrient uptake and biosynthesis of DNA, RNA and proteins. The mechanisms of piezoadaptation are thought to be very complex because organisms are likely to encounter all of the inhibitory effects of high pressure. However, our recent results have revealed that only one gene, *TAT2*, which encodes a tryptophan permease, is sufficient to confer growth of *Saccharomyces cerevisiae* cells at 25 MPa (~250 atm) through its overexpression [1]. We started to isolate high-pressure growth (*HPG*) mutants, with the hope that cloning of the genes would provide insights into the regulation of two tryptophan permeases Tat1 and Tat2. Of the four *HPG* genes identified, the *HPG1* mutation sites are located within the catalytic domain of Rsp5 ubiquitin ligase [2]. Consequently, the two tryptophan permeases are stabilized at high pressure in the mutants. The *HPG2* mutation sites are located in the cytoplasmic tails of Tat2 [3]. Tat1 predominantly localized in the plasma membrane, whereas Tat2 was abundant in the internal membranes. Moreover, Tat1 was associated with sphingolipids-ergosterol rich membrane domain, whereas Tat2 localized in glycerophospholipids. Here, we demonstrate that ubiquitination regulates the turnover, cellular distribution and lipid association of these permeases under high-pressure conditions. Analysis of the activation volume associated with tryptophan uptake suggests that hydrostatic pressure is a unique tool for elucidating the dynamics of integral membrane protein functions as well as probing lipid microenvironments where they localize.

[1] Abe, F. and Horikoshi, K. (2000) *Mol. Cell. Biol.* **20**: 8093-8102.
[2] Abe, F. and Iida, H. (2003) *Mol. Cell. Biol.* **23**: 7566-7584.
[3] Nagayama, A., Kato, C. and Abe, F. (2004) *Extremophiles* **8**:143-149.

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CHARACTERIZATION OF A DPS-LIKE PROTEIN FROM THE HYPERTHERMOPHILIC ARCHAEON *SULFOLOBUS SOLFATARICUS*

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We have characterized a Dps-like protein from the hyperthermophilic archeaon *Sulfolobus solfataricus* (Dps-Ss). The amino acid sequence of this ~19,000 Da protein shares little sequences similarity to proteins with known function. However, the protein shares high sequence similarity to numerous hypothetical proteins in other archaeal and bacterial genomes. Twelve identical subunits of the recombinant protein self assemble into a hollow dodecameric protein cage having 3:2 symmetry. The outer shell diameter is ~9nm and the interior diameter is ~ 5nm. Higher order structural predictions and TEM reconstructions of the assembled Sulfolobus protein cage indicate that it is structurally related to proteins in the ferritin super family and most closely related to a class of DNA binding protein from stayed cells (Dps). Dps proteins have been shown to protect nucleic acids by physically shielding DNA against oxidative damage and by consuming constituents involved in Fenton chemistry. *In vitro* the assembled archaeal protein efficiently uses H_2O_2 to oxidize FeII to FeIII and stores the oxide as a mineral core on the interior surface of the protein cage, while the exterior surface of the cage associates with nucleic acids. The gene is dramatically up regulated in *S. solfataricus* upon oxidative stress, but not induced by various other stresses. The apparent iron-mineralizing function of this protein in addition to its nucleic acid sequestering activity suggest that this archaeal protein may represent an early progenitor of ferritins and Dps family proteins.

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A MULTI-CHAPERONE NETWORK FROM HYPERTHERMOPHILIC ARCHAEA

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Many hyperthermophilic Archaea living at or above 100°C have been well characterized, with increasing access to genomic sequence data that has recently allowed the analysis of the protein families related to stress responses. Although diverse conditions such as heat stress, desiccation, chemical stress or starvation may be the inducers, the effector proteins are usually referred to generically as Heat Shock Proteins (HSPs). Many HSPs function as molecular chaperones and are involved in protein folding pathways. HSPs. can be divided into 3 functional groups, holdases, foldases and unfoldases. Holdases such as the small Heat Shock Protein (sHsp) or prefoldin restrain denatured proteins from aggregation and in some instances deliver soluble proteins to foldases (eg Hsp60s or Hsp70) where the correct quaternary structure is realized. Unfoldases on the other

hand are involved in protein unfolding and protein quality control. Several lines of evidence indicate that chaperones can function cooperatively. We have previously shown that sHsp can maintain aggregation of heat-denatured protein *in vitro*. Recent evidence shows that a multi-chaperone network of chaperones from hyperthermophiles can prevent loss of enzyme activity, for example during the temperature cycling conditions prevailing during PCR reactions. The chaperone activities are compatible with the maintenance of normal functions of DNA polymerases during cycling. Our results also show co-operative functions *in vivo* of the molecular chaperones from the hyperthermophile, *Pyrococcus furiosus* in complementing *E. coli* strains under a variety of stress conditions.

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HIGH SALINITY AND LOW TEMPERATURE INFLUENCE PLASMA MEMBRANE FLUIDITY IN EXTREMOPHILIC FUNGI, ISOLATED FROM HYPERSALINE AND EXTREMELY COLD ENVIRONMENTS

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Low water activity is a limiting factor for organisms inhabiting two different extreme environments - hypersaline waters in the salterns and cold, glacial Arctic environments. Water is bound due to high NaCl concentrations in the first case, while it is unavailable due to the formation of ice crystals in the second. Similarly to hypersaline environments, the extremely cold global regions were considered to be populated almost exclusively by prokaryotic microorganisms. But recently, the methodology used for isolation of fungi from the salterns resulted in the isolation of diverse psychrophilic and psychrotolerant fungi from different types of ice in the Arctics. Different black yeasts species were among the dominating groups of fungi in both environments. Only the genus *Aureobasidium* was found in glacial ice with high ionic strenght as well as in hypersaline waters of solar salterns. The occurrence of the same taxon in two different extreme environments, both characterized by the limited water availability, suggests efficient eco-physiological adaptations and probably similar adaptive strategies. Our research was focused on selected extremophilic black yeast models - *A. pullulans* and *Hortaea werneckii* from the salterns and a new *Aureobasidum* sp. from ice. The influence of low temperature and high NaCl concentration on the lipid composition and the ordering and dynamics of lipid molecules of the plasma membrane was investigated. So far it was discovered that halophilic black yeasts contain a higher ratio of unsaturated phospholipid-esterified fatty acids, a lower sterol-to-phospholipid ratio and as a consequence have more fluid membranes than non-halophilic species of fungi within a broad range of salinities. The comparative study with psychrotolerant fungi, exposed to low temperatures and high NaCl concentrations will be presented. As membrane-mediated processes are central for normal cell function, membrane remodelling is essential for successful adaptation to an altered physicochemical environment and for unaltered physiological cell functions.

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BIOCHEMICAL AND GENETIC CHARACTERIZATION OF THE SYNTHESIS OF MANNOSYLGLYCERATE IN THE GENUS *ARCHAEOGLOBUS*

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Many hyperthermophyles isolated from marine environments accumulate compatible solutes, namely mannosylglycerate (MG), which act as thermoprotectors and osmoprotectors [1]. The strong correlation between the accumulation of MG in response to salt stress in thermophiles and hyperthermophiles with weak phylogenetic relationship suggest that it may be better suited for osmoprotection of organisms growing at high temperatures than are other compatible solutes known to occur in mesophiles [1]. Our results indicate that both *A. veneficus, A. profundus* and *A. fulgidus* 7324 accumulate MG. However, closely related organisms do not accumulate MG, namely the type strain *Archaeoglobus fulgidus* VC-16 in which the homologous genes for the synthesis of MG are absent in the sequenced genome. The pathways leading to the synthesis of MG have been elucidated in a number of euryarchaeotes and bacteria. The most usual route involves the condensation of GDP-mannose and 3-phosphoglycerate yielding a phosphorylated intermediate (MGP), which is then cleaved by a highly specific phosphatase, to produce MG. Based on the known sequences of MGP synthase (MPGS) and phosphatase (MPGP) genes and using PCR and inverse PCR techniques we have identified the genes involved in the synthesis of MG in species of the genus *Archaeoglobus*. As expected due to the close phylogenetic relationship, the MPGS and MPGP from different *Archaeoglobus* species show high degree of identity. We have cloned and functionally overexpressed in *E. coli* both gene sequences and characterized the recombinant enzymes. In *P. horikoshii* the *mpgs* and *mpgp* genes are arranged in an operon-like structure that includes the genes coding for the enzymes that convert fructose-6P into GDP-mannose (M1P-GT/PMI and PMM) [2]. In *T. thermophilus*, *R. marinus* and *A. pernix* the MPGS gene is located immediately upstream of the MPGP gene, but these two organisms lack the genes for M1P-GT/PMI and PMM in adjacent location, indicating a different genomic organization [3, 4, 5]. Interestingly, in *Archaeoglobus* we found the MPGP gene immediately downstream of the MPGS gene, followed by an ORF that presents high homology with á-mannosidases, indicating that *Archaeoglobus* species have a singular operon-like structure. The different organization of the operon-like structures containing the genes involved in MG synthesis and the distribution of this solute in the tree of life are discussed in regards to the possible role of MG in osmo and thermoprotection.

[1] Santos H and da Costa MS (2002). Environ Microbiol 4:501-9.
[2] Empadinhas N, *et al.*; (2001). J Biol Chem 276:43580-8.
[3] Empadinhas N, *et al.*; (2003). Appl Environ Microbiol 69:3272-9.
[4] Borges N, *et al.* (2004). J Biol Chem 279:9892-8.
[5] Kawarabayasi, *et al.* (1999). DNA Res 6:83-101, 145-152.

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NEW PHOSPHORYLATED COMPOUNDS ACCUMULATING IN THE EXTREME HYPERTHERMOPHILE *PYROLOBUS FUMARII*

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One of the general strategies adopted by thermophiles and hyperthermophiles to cope with temperature stress is solute accumulation. In these organisms, supraoptimal conditions of salt and temperature usually bring about a differential accumulation of solutes. Some of these compounds, like mannosylglycerate, tend to accumulate primarily in response to salt stress while the level of di-myo-inositol-1,1'-phosphate increases strongly in response to temperatures above optimal for growth [1-3]. The archaeon *Pyrolobus fumarii* grows optimally above the boiling point of water (106ºC) and is capable of growth up to 113ºC [4]. Moreover, this extreme hyperthermophile is able to withstand autoclaving (1 h at 120ºC) without complete loss of viability. Despite the fact that this remarkable resistance to very high temperatures has been known since 1997, no protein from this organism has been investigated for its stability, nor have general stabilizing strategies (like solute accumulation) been studied. Also, very little is known about the composition of the cell envelope or cytoplasmic membrane, and even less about the biosynthetic pathways leading to their formation. We decided to characterize the compatible solutes of *Pyrolobus fumarii*. After ^{1}H, ^{13}C and ^{31}P-NMR analysis of ethanolic extracts, we identified di-inositol-phosphate as the major solute; in addition, several NDP-sugar like compounds were detected. Using 2D-NMR techniques we identified firmly one of these compounds as UDP-N-2,3 diacetylglucose-diamine. The remaining NDP-sugars are very similar to this one: most of them show differences in the sugar moiety, while one of them has a CDP residue instead of UDP. Thus far, diamino-diacetylated sugar derivatives have been found only as constituents of lipopolysaccharides, namely lipid A, in a few gram-negative bacteria. It is conceivable that these compounds are involved in the synthesis of membrane lipids. Results on the characterisation of the components of the polar lipid fraction will be presented as well.

[1] Martins LO, Santos H; (1995). Appl Environ Microbiol 61:3299-3303.
[2] Martins LO, et al.; (1996). J Bacteriol 178:5644-5651.
[3] Silva Z, et al.; (1999). Extremophiles 3:163-72.
[4] Blochl, et al.; (1997). Extremophiles 1:14-21

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CONCERTED ACTION OF DIACETYLCHITOBIOSE DEACETYLASE AND EXO-ß-D-GLUCOSAMINIDASE IN A NOVEL CHITINOLYTIC PATHWAY IN THE HYPERTHERMOPHILIC ARCHAEON *THERMOCOCCUS KODAKARAENSIS* KOD1

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The hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 possesses chitinase (*Tk*-ChiA) [1, 2] and exo-ß-D-glucosaminidase (*Tk*-GlmA) [3] for chitin degradation, the former produces diacetylchitobiose ($GlcNAc_2$) from chitin and the latter hydrolyzes chitobiose ($GlcN_2$) to glucosamine (GlcN). In order to identify the enzyme that physiologically links these two activities, here we focused on the deacetylase that provides the substrate for *Tk*-GlmA from $GlcNAc_2$. The deacetylase could be detected in and partially purified from *T. kodakaraensis* cells, and the corresponding gene (*Tk-dac*) was identified on the genome. The deduced amino acid sequence was classified into LmbE-protein family including *N*-acetylglucosaminyl-phosphatidylinositol de-*N*-acetylases and 1-D-*myo*-inosityl-2-acetamido-2-deoxy-á-D-glucopyranoside deacetylase. Recombinant *Tk*-Dac showed deacetylase activity toward *N*-acetylchitooligosacchrides ($GlcNAc_{2-5}$), and the deacetylation site was revealed to be specific at the nonreducing GlcNAc residue. The enzyme also deacetylated GlcNAc monomer. In *T. kodakaraensis* cells, the transcription of *Tk-dac*, *Tk-glmA*, *Tk-chiA* and the clustered genes were induced by $GlcNAc_2$, suggesting the function of this gene cluster in chitin catabolism *in vivo*. These results have revealed a unique chitin catabolic pathway in *T. kodakaraensis*, in which $GlcNAc_2$ produced from chitin is degraded by the concerted action of *Tk*-Dac and *Tk*-GlmA. That is, $GlcNAc_2$ is site-specifically deacetylated to GlcN-GlcNAc by *Tk*-Dac and then hydrolyzed to GlcN and GlcNAc by *Tk*-GlmA, followed by a second deacetylation step of the remaining GlcNAc by *Tk*-Dac to form GlcN. This is the first elucidation of an archaeal chitin catabolic pathway, and also defines a novel mechanism for dimer processing using a combination of deacetylation and cleavage, distinct from any previously known pathway [4].

[1] Tanaka, T., Fujiwara, S., Nishikori, S., Fukui, T., Takagi, M., and Imanaka, T. (1999) Appl. Environ. Microbiol. 65, 5338-5344
[2] Tanaka, T., Fukui, T., and Imanaka, T. (2001) J. Biol. Chem. 276, 35629-35635
[3] Tanaka, T., Fukui, T., Atomi, H., and Imanaka, T. (2003) J. Bacteriol. 185, 5175-5181

[4] Tanaka, T., Fukui, T., Fujiwara, S., Atomi, H., and Imanaka, T. (2004) J. Biol. Chem. in press.

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CHARACTERIZATION OF MEMBRANE-BOUND FE(III) REDUCTASE ACTIVITIES FROM THERMOPHILIC GRAM-TYPE POSITIVE DISSIMILATORY IRON-REDUCING BACTERIUM *THERMOTERRABACTERIUM FERRIREDUCENS*

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While the assimilatory ferric reductases have been studied in the variety of microorganisms, the understanding of the enzymes involved in dissimilatory Fe(III) reduction is still very scarce The biochemistry of dissimilatory ferric iron reduction has been investigated intensively only for members of *Proteobacteria* with a Gram-type negative cell wall structure. We have investigated the iron-reducing activity of dissimilatory Fe(III) reducer *Thermoterrabacterium ferrireducens*. Since *T. ferrireducens* has no periplasm the mechanism of Fe(III) reduction is expected to be different from that in gram-negative bacteria. We have found that almost 80% of Fe(III) reductase activity of this organism is localized in the membrane fraction of the cells. This activity was isolated by solubilization of membranes with lauryl maltoside, and partially purified using POROS HQ, CHT hydroxyapatite and DEAE-Sepharose ion exchange chromatography. The main part of the collected chromatographic fractions possessed both NADH- and NADPH-dependent Fe(III) reductase activities in varying ratios but some fractions had almost exclusive NADH- or NADPH dependent activity. After the last purification step the NADH-dependent Fe(III) reductase activity had been enriched 8-fold and the NADPH-dependent one 11-fold, with a yield for both activities of ca. 10%. Native PAGE of purified iron reductase activities stained with ferrozine showed one activity band (ca. 115 kDa) with NADH dependent and two activity bands (ca. 70 and 115 kDa) with NADPH dependent activity. Partially purified Fe(III) reductase activity exhibited both NADH- and NADPH dependent activity when Fe(III)-EDTA (154 mU mg^{-1}), Fe(III) citrate (7.53 mU mg^{-1}) or amorphous Fe(III) oxide (3.5 mU mg^{-1}) were used as an electron acceptor (the maximal NADH dependent activity is shown in parentheses). The temperature optimum of both NADH- and NADPH-dependent Fe(III) reductase activities were about 70°C. UV-Vis spectroscopy of partially purified Fe(III) reductase activity demonstrated the presence of *c*-type cytochromes which could be reduced after the addition of NAD(P)H and subsequently rapidly reoxidized either by soluble Fe(III)-EDTA or by insoluble amorphous Fe(III) oxide. The possibility of cytochromes reoxidation by Fe(III) compounds indicates their involvement in the Fe(III) reductase complex. Our data suggests that Fe(III) reductase of *T. ferrireducens*, being membrane-bound might be involved in energy-generating processes, but in contrast to previously described Fe(III) reductase activity of a Gram-type negative bacterium, the activity of *T. ferrireducens* is probably caused by two enzymes or enzyme complexes with NADH- and NADPH-linked activities. This is the first report on characterization of a dissimilatory ferric reductase activity from a Gram-type positive bacterium.

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ANALYSIS OF SUBSTRATE RECOGNITION BY PAN/20S PROTEASOME ISOFORMS IN THE HALOARCHAEON *HALOFERAX VOLCANII*

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Proteasomes appear to be central to energy-dependent proteolysis in archaea. These enzymes consist of a catalytic 20S proteasome core, which associates with regulators such as the proteasome-activating nucleotidase (Pan), a AAA ATPase. In vitro, substrate binding to Pan activates ATP hydrolysis promoting substrate unfolding. This is presumed to successively open the axial gate and translocate substrate into the 20S proteasome. Much information has arisen from recent efforts to understand the basic mechanism of protein degradation mediated by Pan and the 20S proteasome; however, little of this involves identifying the properties of substrate recognition. To investigate this, a convenient reporter system was developed by functional expression of a green fluorescent protein (GFP) variant with C-terminal fusions in the haloarchaeon *Haloferax volcanii*. An established genetic system along with the unusual synthesis of two Pan proteins (PanA and PanB) made this organism ideal for study. Accumulation of the GFP reporter was dependent on its C-terminal amino acid sequence as well as the addition of an irreversible, proteasome-specific inhibitor (*clasto*-lactocystin-â-lactone) to cell culture. These results provide new insight into how substrate proteins are recognized by proteasomes in archaea. Current research is focused on elucidating the binding affinity of PanA and PanB complexes for these substrate recognition motifs in the presence and absence of 20S proteasomes.

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THE FIRST CHARACTERIZATION OF AN ARCHAEAL GTP-DEPENDENT PHOSPHO-ENOLPYRUVATE CARBOXYKINASE FROM THE HYPERTHERMOPHILIC ARCHAEON, *THERMOCOCCUS KODAKARAENSIS* KOD1

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The hyperthermophilic genera *Thermococcus* and *Pyrococcus*, belonging to the order Thermococcales in *Euryarchaeota*, have been known to utilize a modified Embden-Meyerhof pathway for the degradation of glucose moieties. Alternatively, these archaea generally can grow on amino acids or peptides as carbon and energy sources, where

cellular sugar components are provided via the gluconeogenic pathway. Despite the wealth of knowledge on these pathways, only little information is available with respect to conversion of C3-compounds generated through glycolysis to C4-compounds, and vice versa, in hyperthermophilic archaea. Phosphoenolpyruvate (PEP) carboxykinase (PCK), catalyzing the nucleotide-dependent reversible decarboxylation of oxaloacetate (OAA) to yield PEP and CO_2, is one of the important enzymes in the interconversion between C3 and C4 metabolites. Recently, we have determined the complete genome of a hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1, and the results have enabled us to identify one gene corresponding to an uncharacterized homolog of PCK. This study focused on the first characterization of the enzymatic properties and expression profile of an archaeal PCK from the *T. kodakaraensis* (*Tk*-Pck). *Tk*-Pck showed 30-35% identities to GTP-dependent PCKs from mammals and bacteria, but was located in a distinct branch from the classical enzymes in the phylogenetic tree. Several catalytically important regions, found in all known PCKs irrespective to their nucleotide specificity, were conserved in *Tk*-Pck. However, the predicted GTP-binding region was unique when compared to those in other GTP-dependent PCKs. The recombinant *Tk*-Pck actually exhibited GTP-dependent activity, and was suggested to possess dual cation-binding sites respective for Mn^{2+} and Mg^{2+}. The enzyme preferred PEP formation from OAA, since the K_m value for OAA was much lower than that for PEP. The transcription and activity levels in *T. kodakaraensis* were higher under gluconeogenic conditions than under glycolytic conditions. These results agreed with the function of *Tk*-Pck in providing PEP from OAA as the first step of gluconeogenesis in the hyperthermophilic archaeon. Additionally, under gluconeogenic conditions, we observed higher expression levels of *Tk*-Pck on pyruvate than on amino acids, implying an additional role in the recycling of excess PEP produced from pyruvate, replacing the function of the anaplerotic PEP carboxylase that is missing in this archaeon.

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NEW EVIDENCE FOR SULPHUR METABOLISM IN HYPERTHEROPHILIC BACTERIA AQUIFEX AEOLICUS : BIOCHEMICAL AND PHYSIOLOGICAL STUDIES

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Microorganisms having the remarkable property of growing at temperatures near and above 100°C have been isolated from shallow submarine and deep-sea volcanic environments over the last 20 years. In the bacteria domain, the most hyperthermophilic organisms known to date are member of the genus Aquifex, which forms the lowest branching order of the domain Bacteria. The full genome of a hyperthermophilic representative, Aquifex aeolicus, has been sequenced a few years ago. Aquifex is a hyperthermophilic, hydrogen-oxidizing microaerophilic, obligate chemolithoautroph. Aquifex aeolicus is usually cultured at 85°C under H2/CO2/O2 (79.5:19.5:1) atmosphere in a medium containing only inorganic components. To date, experiments were performed on these standard conditions. We have recently characterized cytochrome bc1 complex, membranous and soluble cytochromes c555 and two of the three hydrogenases annotated in the genome from Aquifex aeolicus. Due to the observation that a close relative of Aquifex aeolicus, Aquifex pyrophilus can use S° as electron acceptor and that in the sequenced genome of Aquifex aeolicus several genes might be involved in sulphur metabolism, cultures were performed by replacing sodium thiosulfate by sulphur. We have shown that Aquifex aeolicus can use sulphur as electron acceptor. By a physiological approach, a variation in the hydrogenase content of Aquifex aeolicus has been demonstrated depending on the growth phase and the medium used (standard medium or sulphur. Hydrogenase III was constitutive in our experiments conditions and might be involved in providing the reducing power necessary for driving the TCA cycle in reverse. Hydrogenases I and II production and activities depended differentially on the growth phase and the medium used. In parallel, we studied proteins which should be expressed under sulphur conditions. Two important groups of enzymes are known to play a crucial role in the sulphur metabolism: the sulphur reductases and the sulphur transferases (rhodaneses). A membrane-bound sulphur reducing complex transferring electron from the H2 to the S° has been purified. Twelve proteins were identified and the majority are known to be involved, in Aquifex or other organisms, in electron transport chains or in energy conservation. The cytoplasmic localisation of the sulphur reductase is not consistent with the substrate accessibility but it was shown previously that electron transport from H2 to sulphur was stimulated by the presence of sulphur transferase. Five sulphur transferases (rhdA1, rhdA2, Aq477, Aq1599 and Aq2056) have been shown to be present on sulphur conditions. Products from Aq477, Aq1599 and Aq2056 genes have been purified. Aq1599 and Aq477, Aq2056 have been localized in the periplasmic and cytoplasmic space, respectively. From all these results we propose a model for sulphur respiration in Aquifex aeolicus.

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MUTATIONAL ANALYSIS OF THR ACTIVE SITE OF THE *B*-PROTEIN IN THE 20S PROTEASOME SUGGESTS *B*-PROTEIN PROCESSING VIA AN INTRAMOLECULAR MECHANISM

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Proteasomes are energy-dependent proteases found in all domains of life. The 26S proteasome is formed from a 20S particle and 19S cap, where the 20S particle is responsible for catalytic activity. The 20S proteasome is a cylindrical barrel composed of four stacked heptameric rings. The two outer rings consist of seven á-type subunits, while the two inner rings consist of seven â-type subunits. The â-type subunits are involved in peptide cleavage mediated by an NH_2-terminal Thr residue that is responsible for the nucleophilic attack of peptide bonds. An NH_2-terminal propeptide is autocatalytically cleaved to expose the active site Thr residue during the final stages of 20S proteasome maturation. In eukaryotes and archaea, assembly of the 20S proteasome is thought to occur in two steps: the formation of a preproteasome complex, where the á-rings and unprocessed â-rings combine, and finally the combination of two half-proteasomes dimerizing at the â-ring interface to form a 20S

complex. Cleavage of the â-propeptide yields a mature 20S proteasome. To date, *Haloferax volcanii* is the only archaeon in which the assembly of 20S proteasomes can readily be examined *in vivo*. Currently it is unclear whether the â-subunits of archaeal proteasomes are processed via an inter- or intra-molecular mechanism. In this study, the Thr residue of the â-protein, which forms the active site, was modified to Ala and Ser along with the addition of a C-terminal polyhistidine tag. These modified â-proteins (â-His, â-Thr49Ala-His, â-Thr49Ser-His) were separately expressed in *Haloferax volcanii* and purified by Ni^{2+}-Sepharose chromatography. Protein fractions were analyzed by Western blot using polyclonal antibodies raised against á1, á2, and â. The results revealed that all three â-His tagged proteins assembled with the á1, á2, and â subunits expressed from the genome. The â-subunit expressed from the genome was processed in all three samples; however, differences in the extent of â-His tagged protein processing were observed. The â-His protein was completely processed. In contrast, the Thr49Ser mutant displayed limited â-processing, while the Thr49Ala mutant showed no processing. These results suggest that processing is occurring by an intramolecular mechanism.

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CARBOXYDOCELLA SPOROFORMA SP. NOV., A NOVEL ANAEROBIC, THERMOPHILIC, CO-UTILIZING HYDROGENOGENIC BACTERIUM FROM A KAMCHATKA HOT SPRING

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A novel anaerobic, thermophilic, CO-utilizing bacterium, strain Kar, was isolated from a terrestrial hot spring of Karymskoe Lake, Kamchatka Peninsula. The cells of the new isolate were Gram-positive sporeforming short rods. The bacterium grew chemolithoautotrophically on CO, producing equimolar quantities of H_2 and CO_2 (according to the equation $CO + H_2O$! $CO_2 + H_2$), and in the absence of CO, under N_2 in the gas phase, organotrophically with yeast extract, sucrose or pyruvate as the energy substrates. The isolate did not grow on peptone, starch, cellobiose, glucose, arabinose, fructose, xylose, galactose, lactose, maltose, glycerol, acetate, citrate, succinate, formate, ethanol and methanol. H_2:CO_2 gas mixture (4:1, v/v) did not support the growth. No growth was observed on different organic substrates in the presence of sulfate, thiosulfate, elemental sulfur or nitrate. The microorganism did not grow on solid media; it was able to grow only in the semisolid medium with 0.5% agar. Growth was observed in the temperature range from 50 to 70°C, with an optimum at 60°C, and in the pH range from 6.2 to 8.0, with an optimum at pH 6.8. Growth was completely inhibited by penicillin, novobiocin, streptomycin, kanamycin, and neomycin. The generation time under optimal conditions for chemolithoautotrophic growth was 1 h. G+C content of the DNA 49.5±1 mol.%. The analysis of 16S rDNA sequence showed that the isolate shoud be assigned to genus *Carboxydocella*. It differs from *Carboxydocella thermautotrophica* by the lack of motility, spore formation and ability of organotrophic growth, thus, representing, most probably, a second species of the genus *Carboxydocella*.

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NEW ANAEROBIC THERMOPHILIC PROCARYOTES WITH HYDROLYTIC ACTIVITIES

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24 samples from Kamchatka and Baikal region hot springs, Russia, with temperature ranges from 35 to 80°C and pH from 2.0 to 9.6, were screened for the presence of anaerobic microorganisms with hydrolytic activities. Substrates were insoluble carbohydrates such as starch, xylan, chitin, agarose, cellulose and carboxymethyl cellulose and proteins such as alpha- and beta-keratins, albumin, gelatin and casein. pH of the media and the incubation temperature were similar to conditions in sites of the isolation. From stable enrichments several new isolates were obtained. Two strains of moderately thermophilic and moderately acidophilic rod-shaped anaerobic bacteria were isolated. Both organisms were able to grow on different soluble carbohydrates, as well as on starch and albumin. Strain 761 grew optimally at 55°C and at pH 5.7 and was found to represent a novel species of the genus *Thermoanaerobacterium*, while strain 711 had its growth optima at 60°C and pH 5.0, was representing the genus *Thermoanaerobacter*. Strain 1004 had rod-shaped cells 2-10 ìm long and 0.6 ìm wide and grew optimally at 65°C and at pH 6.8. This isolate apart of growth on soluble substrates was able to hydrolyze feather and pork-wool keratins, casein, gelatin and albumin. After four days of cultivation its cell yield achieved $1.9x10^8$ cells ml^{-1} when the feather keratin was used as the growth substrate and $3.8x10^8$ cells when growing on albumin. Analysis of 16S rDNA sequence revealed its affiliation to the genus *Thermoanaerobacter* where it represented a new species. This microorganism produced extracellular keratinase with the molecular weight about 150 kDa. The characterization of enzyme is in progress. Another new strain 940-1 grew at temperature 70°C and pH 6.1. The cells of this organism were long, thin filaments with Gram-negative cell wall, often aggregated in irregular spherical structures. It grew on diverse sugars and polysaccharides, including cellulose and carboxymethyl cellulose. Phylogenetic position of isolate 940-1 is under study.

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BIODEGRADATION OF AROMATIC COMPOUNDS BY EXTREMELY HALOPHILIC MICROORGANISMS

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Extremophiles offer increasingly new opportunities for the isolation of novel biomolecules that have the potential to greatly promote

biotechnology. They are a source of extremely stable enzymes (extremozymes), which have the possibility to directly, through first hand use, or indirectly, through enzyme engineering, benefit biocatalysis. The extremely halophilic archaea are one group that we are predominantly interested in. In particular it is the stability of their proteins in high salt (effectively a low water environment), which makes them potentially very interesting biosubstances for industry. Towards this end we have isolated and characterised novel aromatic and amide degrading haloarchaea, particularly those that possess oxygenase-mediated degradation routes. We have determined the pathways by which they are metabolised and the enzymes and genes responsible. We present here data on recently isolated strains capable of degrading a number of aromatic compounds, and compare these to previous haloarchaeal strains isolated here. We also include data on investigating the possibility of CoA-mediated aerobic aromatic catabolic pathways. Furthermore we present a novel method for synthesizing aromatic CoA thioesters for use as substrates in enzyme assays.

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PHYSIOLOGICAL CHARACTERIZATION OF A PROTEASOME-DEFICIENT MUTANT OF *HALOFERAX VOLCANII* BY A DIFFERENTIAL PROTEOMIC APPROACH

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Halophilic archaea (haloarchaea) flourish in hypersaline (3 - 5 M) environments by maintaining an osmotic balance through the exclusion of sodium ions and accumulation of potassium as an internal compatible solute. Progress in proteomics and functional genomics has facilitated the use of haloarchaea as simple models for many key cellular processes, including the control of information (DNA replication, transcription, translation) and protein turnover/quality (proteasomes, chaperonins). Many of these basic mechanisms are more closely related in archaea to their eucaryal than eubacterial counterparts. To further understand the physiological role of the proteasome, a proteasome-activating nucleotidase mutant (*panA*) was constructed in the haloarchaeon *Haloferax volcanii*. Reproducible 2D-PAGE proteome maps generated by microrange IPG strips (3.9-5.1) of wild type cells were compared to an isogenic *panA* mutant (DS70 vs. GG102). A triplet of protein spots with the same molecular mass (41 kDa) but different pI values (4.41, 4.36 and 4.31) were found to be unique to the *panA* mutant. QSTAR LC/MS/MS coupled with *de novo* sequencing of tryptic fragments identified these spots as the same protein: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) of *Haloarcula hispanica* of which the encoding gene was used as a mevinolin-resistance marker to construct the *panA* mutation. The separation of HMGR into three isoforms by isoelectric focusing suggested post-translational modification of this metabolic enzyme, which is a catalyst for the rate-limiting reaction in the biosynthesis of isoprenoids. Consistent with this, all three HMGR isoforms were detected by differential phosphoprotein staining (ProQ Diamond). Prior to our work, only the eucaryal HMGR appeared to be phosphorylated in vivo. Thus, current efforts are underway to define the phosphorylation sites of these archaeal HMGR isoforms that accumulate in the proteasome-activating nucleotidase (*panA*) mutant.

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TRYPTOPHAN PERMEASE TAT2 IS ESSENTIAL FOR HIGH-PRESSURE GROWTH IN SACCHAROMYCES CEREVISIAE MUTANTS DEFECTIVE IN UBIQUITIN-SPECIFIC PROTEOLYSIS

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Our recent results indicate that tryptophan uptake is a limiting factor for cell growth of the yeast Saccharomyces cerevisiae under high pressure and low temperature conditions (1). High-pressure incubation of cells causes degradation of tryptophan permease Tat2, which is mediated by Rsp5 ubiquitin ligase (2). The HPG (high-pressure growth) 1 mutation is allelic to RSP5 and the mutation stabilizes Tat2 in the plasma membrane (2). In this paper, we show that deubiquitination step has a role in pressure-induced degradation of Tat2. Ubiquitinated proteins are subjected to ubiquitin-specific proteolysis prior to degradation by the 26S proteasome or the vacuoles. Mutation or deletion of ubiquitin-specific protease genes (UBP) is known to produce pleiotropic effects on sensitivity to a variety of environmental stresses. Of the 17 ubp mutants, doa4, ubp6 and ubp14 cells exhibited growth at 25 MPa and 15 °C. Indeed, Tat2 was stabilized in these ubp mutants. The result suggests that the three Ubp proteins have a role in the degradation of Tat2 at high pressure. Internal free ubiquitin level is known to decrease upon the ubp mutations. However, overexpression of ubiquitin did not inhibit their high-pressure growth. Therefore, multiple deubiquitination steps through Doa4, Ubp6 and Ubp14 rather than free ubiquitin depletion are likely to be involved in Tat2 degradation at high pressure.

References

(1) Abe, F. and Horikoshi, K. (2000) Mol. Cell. Biol. 20, 8093-8102.
(2) Abe, F. and Iida, H. (2003) Mol. Cell. Biol. 23, 7566-7584.

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TAQ DNA POLYMERASE ASSUMES AN ELONGATED CONFORMATION IN SOLUTION

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Escherichia coli "Pol 1" and *Thermus aquaticus* "Taq" are homologous Type I DNA polymerases, each comprised of a polymerase domain, a proofreading domain (inactive in Taq), and a 5' nuclease domain. "Klenow" and "Klentaq" are the large fragments of Pol 1 and Taq and are functional polymerases lacking the 5' nuclease domain. In the available crystal structures of full-length Taq, the 5' nuclease domain is positioned in two different orientations: in one structure it is extended out into solution, while in the other it is folded up against the polymerase domain in a more compact structure. These

different conformations lead to distinct functional hypotheses. The more compact structure could allow both the polymerase and 5'nuclease activities of the same polymerase molecule to act within relative proximity on the same piece of DNA. The elongated structure would require greater separation of the substrate sites on the DNA, or may even suggest that two polymerase molecules are required for simultaneous polymerase + 5'exonuclease activity. Analytical ultracentrifugation and small angle x-ray scattering (SAXS) were used to study the global conformations of the polymerases in solution. $S_{20,w}$ values and partial specific volumes for each polymerase were measured by analytical ultracentrifugation. The $s_{20,w}$ values for full-length Taq and Pol 1 are both consistent with an elongated conformation. The measured partial specific volumes are quite similar for both species of polymerase, indicating no significant differences in packing density between the mesophilic and thermophilic proteins. Radii of gyration measured by small angle x-ray scattering were compared with predicted scattering properties of the polymerases calculated directly from the different crystal structures. The combined experimental and computational characterizations consistently indicate that the full-length polymerases in solution are in a conformation where the 5'nuclease domain is extended into solution. Additional SAXS studies indicate that the radius of gyration of full-length Taq is not altered upon binding primed-template DNA or ddATP. Using approaches devised by Dimitri Svergun and associates, we have also created an envelope-like model of the full-length Taq DNA polymerase directly from the scattering data without using information from the crystal structure. This model of Taq polymerase bares a remarkable resemblance to the elongated Taq crystal structure.

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MULTIENZYME COMPLEXES IN HALOPHILIC ARCHAEA

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Aerobic bacteria and eukarya posses a family of 2-oxoacid dehydrogenase multienzyme complexes (OADHC), which function in pathways of central metabolism for the oxidative decarboxylation of 2-oxoacids yielding acyl CoA and NADH. The complexes consist of multiple copies of three enzymes (E1, E2 and E3). In contrast, no 2-oxoacid dehydrogenase complex activities have ever been found in Archaea; alternative 2-oxoacid ferredoxin oxidoreductases are found throughout the Archaea and in anaerobic bacteria. Therefore, it was a surprise to detect both DHlipDH (E3) activity in a variety of archaea (1) and gene sequences corresponding to a 2-oxoacid dehydrogenase multienzyme complex operon with no known functional significance. Northern analysis of Haloferax volcanii RNA strongly indicates that the whole operon is transcribed as a single message in both aerobically and anaerobically grown cells (3). The detection of ODHC genes in the genomes of both halophilic and thermophilic Archaea adds further weight to the idea that archaeal metabolism shares many common features with that of bacteria and eukarya, and that the pathways of central metabolism may have been laid down before the divergence of the three domains (2). Therefore we have chosen to investigate the expression and function of E3 and multienzyme complexes in halophiles.

·Are the E1 and E2 components also active, and do the components assemble into a functional multienzyme complex?

·Does the complex have an alternative substrate that is not found in non-archaeal organisms?

In vivo expression of DHlipDH was confirmed by growth studies on a variety of substrates but there was no change in the level of expression . No complex activity could be detected with pyruvate as the 2-oxoacid substrate. An alternative approach is to investigate transcription of the whole operon and/or individual genes by using RT-PCR. The results of the growth studies and transcription studies will be presented.

1.Danson, M. J., Eisennthal, R., Hall, S., Kessell, S. R. and William, D. L. (1984). Dihydrolipoamide dehydrogenase from halophilic archaebacteria: purification and properties of the enzyme from Halobacterium halobium. Biochemistry USA, 25, 3880-3884.

2.Danson, M. J., Russell, R. J. M., Hough, D. W. and Taylor, G. L. (1998). Comparative enzymology as an aid to understanding evolution. In thermophiles: the keys to molecular evolution and the origin of life?, 255-267. Edited by M.W.W.Adams and J. Wiegel.London: Taylor and Francis.

3.Jolley, K. A., Maddocks, D. G., Gyles, S. L., Mullan, Z., Tang, S., Dyall-Smith, M.L., Hough, D. W. and Danson, M. J. (2000). 2-Oxoacid dehydrogenase Multienzyme complexes in the Halophilic Archaea? Gene sequences and protein structural predictions. Microbiology, 146, 1061-1069.

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CLONING, OVEREXPRESSION AND CHARACTERISATION OF NOVEL THERMOPHILIC NITRILASE ENZYMES

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By virtue of their enhanced stability towards extreme temperature, pH and solvents, the enzymes from extremophilic organisms are becoming recognized as robust catalysts that are potentially useful for industrial applications. Nitrilases (E.C. 3.5.5.1) catalyse the hydrolysis of nitrile compounds directly to the corresponding carboxylic acid in the absence of the intermediate amide that is produced in the two-step nitrile hydratase/amidase reaction. The most extensively studied nitrilase is that of the soil bacterium Rhodococcus rhodochrous, however, this nitrilase is used as a whole cell preparation for biocatalysis since the isolated enzyme shows limited stability. A BLAST search using the nitrilase gene sequence from R. rhodochrous identified several putative nitrilase-encoding genes within the genomes of thermophilic organisms. Genomic DNA isolated from two of the thermophilic archaea highlighted in the search was used as a template for polymerase chain reaction to amplify three putative nitrilase-encoding genes. Addition of restriction enzyme sequences onto the 5'- and 3'-ends of the genes enabled ligation into the pTrc expression vector system. Subsequent over-expression of one of the genes produced protein that corresponded to the expected molecular weight of approximately 30kDa and proved active towards the substrate benzonitrile. Purification of this protein has been achieved with activity towards

the substrate benzonitrile maintained throughout. This nitrilase has been biochemically characterized and substrate specificity investigated. Since there is no known structure for a nitrilase enzyme from any source, production of diffraction quality crystals for the thermophilic nitrilases is underway. The resultant crystal structures will provide information as to the properties and mechanism of these enzymes.

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THERMODYNAMIC CHARACTERIZATION OF THE HIGH TEMPERATURE STABILITY OF *THERMUS AQUATICUS* DNA POLYMERASE I

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We have examined the chemical and thermal denaturations of the large fragment domains of the Type 1 DNA polymerases from *Thermus aquaticus* (Klentaq) and *E. coli* (Klenow) in order to directly compare the stabilization energetics of the two proteins. The thermal stability of Taq polymerase is common knowledge, and is the basis of its use in the polymerase chain reaction (PCR). However, the thermodynamic and molecular bases for the thermal stability of Taq are just beginning to be elucidated. Chemical denaturations report a stabilization free energy (ÄG) for Klentaq that is over 20 kcal/mole more favorable than that for Klenow at 25°C. This difference between the stabilization free energies of a homologous mesophilic-thermophilic protein pair is significantly larger than generally observed. This is due in part to the fact that the stabilization ÄG for Klentaq polymerase, at 27.5 kcal/mole, is one of the largest ever determined for a monomeric protein. While chemical denaturation of Klentaq is reversible, its thermal denaturation is not, and both calorimetric and optically monitored thermal denaturation of both polymerases indicate that they unfold in a two-state equilibrium process, followed by an irreversible kinetic step. Klenow denatures, in the absence of salt, with a Tm near 40°C, while Klentaq melts at 100°C. At their respective melting temperatures, both the ÄH of unfolding and the kinetic activation barrier (Ea) for irreversible denaturation are significantly larger for Klentaq than Klenow. In order to compare the ÄH and ÄS of unfolding of the two proteins at the same temperature, the heat capacity of unfolding (ÄCp) must be determined. We have measured the stabilization ÄG for both proteins as a function of temperature using chemical denaturation and analyzed the results using the Gibbs-Helmholtz equation to obtain a ÄCp of unfolding. We find that Klentaq has a higher ÄCp of unfolding than Klenow (4.4 kcal/mole K versus 3.1 kcal/mole K). This finding is contrary to most mesophilic-thermophilic protein pairs, where the thermophilic partner usually exhibits the lower ÄCp. Lower ÄCp values have been proposed to be correlated with residual structure in the denatured state of the thermophilic partner in mesophilic-thermophilic protein pairs. Although the native states of Klenow and Klentaq are very similar in size and structure, analytical ultracentrifugation and dynamic light scattering indicate that their denatured states differ in size.

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TRYPSIN Y MAY BE THE DIGESTIVE ENZYME PRODUCED IN ATLANTIC COD UNDER COLD-SHOCK CONDITIONS

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Atlantic cod (*Gadus morhua*) trypsin Y is one of eight novel group III trypsins characterized from their cDNA sequences. The group III trypsins evolved relatively recently from other trypsins possibly due to selective pressure such as extreme cold adaptation (Roach, 2000). Prior to the data presented here, none of the group III trypsins had been isolated in their native form and characterized. The recombinant form of trypsin Y is the only group III trypsin expressed in microorganisms and characterized with respect to biochemical parameters (Pálsdóttir and Gudmundsdóttir, 2004). Interestingly, amino acid sequence alignments as well as amino acid composition comparisons show that the group III trypsins have trends that are approximately opposite to the traditionally classified cold-adapted group I trypsins such as Atlantic cod trypsin I and salmon (*Salmon salar*) trypsin I. Most of these differences are found in surface loops. We describe the isolation of native trypsin Y from Atlantic cod pyloric ceca using polyclonal antibodies raised towards the recombinant enzyme and its purification with known chromatography methods. Our results demonstrate that the native- and recombinant forms of trypsin Y appear to be identical. Thus, trypsin Y has a dual substrate specificity demonstrating both trypsin and chymotrypsin activities. It shows increasing proteolytic activity at temperatures between 2-21°C and the enzyme is completely inactivated at 30°C. Trypsin Y may therefore be considered to be a "super active" enzyme according to the classification of Roach (2000). Also, it may be the digestive enzyme produced under cold-shock conditions as previously suggested (Spilliaert and Gudmundsdóttir, 1999; Roach, 2000). The validity of this theory is currently being tested in our laboratory, by monitoring the expression of trypsin Y in Atlantic cod grown at two different environmental temperatures (2°C and 10°C). The novel properties of trypsin Y may be important for its commercial use. Other cold-adapted proteolytic enzymes from the Atlantic cod have proven their usefulness in industry and medicine. The potential use of cod trypsins I and Y as biomarkers to evaluate the nutritional status of larvae in cod farming are being analyzed in our laboratory as trypsins play a key role in fish development.

Pálsdóttir, H.M., and Gudmundsdóttir, Á. (2004). J. Aquat. Food Prod. Technol. 13(2):85-100.
Roach, J.C. (2002). Proteins 47: 31-44.Spilliaert, R., and Gudmundsdóttir, Á. (1999). Marine Biotechnol. 1: 598-607.

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BIOCHEMISTRY OF DENITRIFICATION IN THE ARCHAEON PYROBACULUM AEROPHILUM

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The pathway of denitrification in the hyperthermophilic P. aerophilum is significantly distinct from that in mesophilic Bacteria. This pertains to the location, type of electron donor and the structural properties of the four enzymes involved. Some of the differences seem to reflect specific adaptations to life at high temperatures. We have determined that all four enzymes involved in denitrification in Pyrobaculum aerophilum, nitrate reductase (NAR), nitrite reductase (NIR), NO reductase (qNOR) and N2O reductase are membrane-bound and utilize menaquinol as electron donor. This is in contrast to e.g. Gram-negative bacteria where NIR and N2O reductase are periplasmic soluble proteins using cytochromes and blue copper proteins as electron donors. A further difference is the lack of membrane bound NADH dehydrogenase activity in P. aerophilum. In stead, a formate dehydrogenase of the Fdh-N type serves as the reductant for the menaquinone pool. In addition, nitrate reduction occurs in the 'periplasmic' space and not in the cytoplasm; as a consequence no energy is conserved in this step and neither nitrate nor nitrite need to traverse the cytoplasmic membrane in accordance with the absence of a NarK homolog from the genome. Up to now we have purified and characterized NAR, NIR and qNOR. While all are metallo-redox enzymes as in Bacteria, the properties in which they differ from their bacterial counterparts are listed below. P. aerophilum lacks heme b but contains the modified hemes Op1 and Op2, containing an ethenylgeranylgeranyl and hydroxyethylgeranylgeranyl modification, respectively. These hydrophobic side chains might engage in extra hydrophobic contacts leading to compaction and stabilization of the protein structure explaining half lives at 100C of 90 min or longer. In qNOR, hemes Op1 and Op2, are present in a 1:1 ratio. EPR and other spectroscopic studies indicate that the enzyme contains a μ-oxo bridged heme Fe-non-heme Fe active site. The NIR contains a modified form of heme d1; also the covalently bound 'c-type heme' may contain a hydrophobic tail. NIR is a two subunit membrane bound glycoprotein, glycosylation occurring in the c subunit. The N-terminal sequence of the d1 subunit contains a motive for translocation via the TAT pathway (..RRDLLK¼) followed by a hydrophobic stretch of 17 amino acids. This sequence is different from the consensus motive in Sulfolobus solfataricus RRXFLK. NAR consists of three subunits. The main heme in NAR (70%) is heme Op2. It further contains 20 Fe atoms in different iron-sulphur clusters three of which are distinguished by EPR, and two pterin-GMP residues. The pterin of the enzyme can accommodate either Mo or W, dependent on the [Mo] or [W] in the growth medium. Interestingly, both the Mo- and W-containing NAR are active indicating the adapative capacity of the organism. Other catalytic, structural and spectroscopic data will be presented as well.

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STRUCTURE AND FUNCTION OF MOLECULAR CHAPERONES FROM HYPERTHERMOPHILIC ARCHAEA

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Molecular chaperones are ubiquitous proteins that are required for the correct folding, assembly, transport and degradation of proteins within the cell. Most of them belong to heat shock protein families. Hyperthermophilic archaea are also equipped with molecular chaperones to protect their proteins from various stresses. Compared with other organisms, the molecular chaperone system of them is simple. Among major chaperone families, only the genes for sHsp and Hsp60 were found in the total genomic sequences of hyperthermophilic archaea. We have been studying structure and function of the principal members of the hyperthermophilic archaeal molecular chaperone system, sHsp, group II chaperonin and prefoldin, a cofactor the chaperonin. We are going to present the recent results on the following subjects of the archaeal molecular chaperones.

1) Structural and functional characterization of sHsps from the thermoacidophilic crenarhaeon, *Sulfolobus tokodaii* strain 7

2) Protein folding mechanism by the group II chaperonin from *Thermococcus* sp. strain KS-1.

3) Physical interaction and functional cooperation between prefoldin and chaperonin.

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A NEW THERMOPHILIC AND THERMOSTABLE ESTERASE WITH SEQUENCE SIMILARITY TO HORMONE-SENSITIVE LIPASE FAMILY FROM A METAGENOMIC LIBRARY

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A gene coding for a thermostable esterase was isolated by functional screening of *Escherichia coli* cells that were transformed with fosmid environmental DNA libraries constructed with the metagenomes from thermal environment samples. The gene conferring esterase activity on *E. coli* grown on tributyrin agar was composed of 936 bp, corresponding to 311 amino acid residues with a molecular mass of 34 kDa. The enzyme showed a significant amino acid similarity (64%) to the enzyme from hyperthermophilic archaea *Pyrobaculum calidifontis*. An amino acid sequence comparison with other esterases and lipases revealed that the enzyme should be classified as a new member of the hormone-sensitive lipase family. The recombinant esterase that was over-expressed and purified from *E. coli* showed maximal activity at 95°C and had high thermal stability. It displayed a high degree of activity in a pH range of 5.5 to 7.5 with an optimal pH of approximately 6.0. The best substrate for the enzyme among the *p*-nitrophenyl esters (C_4-C_{16}) examined was *p*-nitrophenyl caproate (C_6), and no lipolytic activity was observed with esters containing an acyl chain length of longer than 10 carbon atoms,

indicating that the enzyme is an esterase ant not a lipase. This is the first thermophilic and thermostable esterase isolated from a thermal environment metagenomic library.

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EXPLORATION OF THE CATALYTIC MACHINERY OF THE ALPHA-L-FUCOSIDASE FROM SULFOLOBUS SOLFATARICUS

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We have recently reported that a functional a-L-fucosidase could be expressed by a single insertional mutation in the region of overlap between the ORFs SSO11867 and SSO3060 of the hyperthermophilic Archaeon Sulfolobus solfataricus [Cobucci-Ponzano et al. 2003a]. This enzyme, belonging to glycoside hydrolase family 29 (GH29), showed micro-molar specificity for p-nitrophenyl-a-L-fucoside (pNp-Fuc) and promoted transfucosylation reactions by following a reaction mechanism in which the products retained the anomeric configuration of the substrate. We unequivocally identified the catalytic nucleophile of the reaction by reactivation with sodium azide of the mutant Asp242Gly that shows a 10,000-fold activity reduction on pNp-Fuc. In fact, detailed stereochemical analysis of the fucosyl-azide produced by the reactivated mutant revealed its inverted (beta-fucosyl azide) configuration. This allows for the first time the unambiguous assignment of Asp242 as the catalytic nucleophile of the a-L-fucosidase [Cobucci-Ponzano et al. 2003b]. This is the first time that this approach is used for a-L-glycosidases, widening the applicability of this method. To identify other amino acids involved in the mechanism of reaction as acid/base catalyst or with different functions we identified highly conserved residues in GH29 and we prepared the mutants His46Gly, Glu58Gly, His123Gly, Asp124Gly, Asp146Gly, and Glu292Gly. All the purified mutants were affected in their catalytic efficiency, thermal stability or both suggesting that the mutated residues play a relevant role in catalysis and/or in the thermal stabilization of the enzyme. In particular, the mutants Glu58Gly and Glu292Gly showed impaired activity on pNp-Fuc and, for the former, reactivation in the presence of external ions, suggesting for these residues a possible role as acid/base catalysts. The detailed enzymatic characterization of all the mutants and their possible roles in catalysis will be discussed.

Cobucci-Ponzano B. et al. J. Biol. Chem. 2003a, 278, 14622-14631
Cobucci-Ponzano B. et al. Biochemistry. 2003b, 42, 9525-9531.

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IDENTIFICATION AND CHARACTERIZATION OF *SULFOLOBUS SOLFATARICUS* D-GLUCONATE DEHYDRATASE: A KEY ENZYME IN THE NONPHOSPHORYLATED ENTNER-DOUDOROFF PATHWAY

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The extremely thermoacidophilic archaeon *Sulfolobus solfataricus* utilizes D-glucose as sole carbon and energy sources via nonphosphorylated Entner-Doudoroff pathway. It has been suggested that this microorganism metabolizes D-gluconate, the oxidized form of D-glucose, to pyruvate and D-glyceraldehyde by two unique enzymes, D-gluconate dehydratase and 2-keto-3-deoxy-D-gluconate aldolase. In this study, we report the purification and characterization of D-gluconate dehydratase from *S. solfataricus*, which catalyzes the conversion of D-gluconate to 2-keto-3-deoxy-D-gluconate. D-Gluconate dehydratase was purified 400-fold from extracts of *S. solfataricus* by ammonium sulfate fractionation and chromatography on DEAE-Sepharose, Q-Sepharose, Phenyl Sepharose, and Mono Q. The native protein showed a molecular weight of 350 kD on gel filtration. Sodium dodecyl sulfate-polyacrylamide gels gave a molecular weight of 44 kDa indicating that D-gluconate dehydratase is an octameric protein. The enzyme showed maximal activity at temperatures between 80 and 90°C and the pH between pH 6.5 and 7.5. The half-life of the enzyme was 40 min at 100°C. The divalent metal ions such as Co^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+} were activators while EDTA inhibited enzyme activities. Of 22 aldonic acids tested, only D-gluconate served as a substrate. From N-terminal sequences of the purified enzyme, it was found that the gene product of SSO3198 in the *S. solfataricus* genome database corresponds to D-gluconate dehydratase (*gnaD*). In the present study, we have also shown that the D-gluconate dehydratase of *S. solfataricus* is a phosphoprotein whose catalytic activity is regulated by phosphorylation-dephosphorylation mechanism. This is the first report on biochemical and genetic characterization of the D-gluconate dehydratase involved in the nonphosphorylated Entner-Doudoroff pathway.

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GLYCOSYLATION OF *METHANOCOCCUS VOLTAE* FLAGELLINS: STRUCTURAL CHARACTERIZATION AND MUTANT STUDIES

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The flagellin proteins from many Archaea species have been shown to be post-translationally modified through glycosylation. However, very few glycan structures have been determined for these modifications and the underlying pathway leading to glycosylation within Archaea remains unknown. Using the archaeon *Methanococcus voltae*, which possesses 4 flagellin proteins with a combined total of 15

potential N-linked glycosylation sites, we carried out a combination of nLC-MS/MS, accurate mass analysis, MS/MS and NMR to identify the exact nature of the glycan moieties. An N-linked trisaccharide was identified at 14 of the 15 possible sites, with an N-acetyl glucosamine (GluNAc) residue directly linked to asparagine, while the two distal sugars characterized by NMR appear to be modified uronic acids. We then targeted 5 genes and gene clusters of the *M. voltae* genome that may be involved in flagellin glycosylation for mutagenesis studies. These genes include 2 GluNAc transferases (one homologous to *pglC* from *Campylobacter jejuni* and one predicted to be involved in lipopolysaccharide synthesis), an oligosaccharyl transferase STT3 subunit (homologous to *pglB* from *C. jejuni*), an abequose synthase and a heteropolysaccharide repeat unit export protein. *M. voltae* mutants were generated by the insertional inactivation of each targeted gene by the integration of a plasmid carrying an internal fragment of the gene as a site for homologous recombination. Mutants were confirmed by Southern blot analysis and flagellin proteins were characterized by western blotting. Of the mutants generated, only the lipopolysaccharide GluNAc transferase mutant had a detectably altered flagellin phenotype. This mutant was non-motile and did not possess flagella when examined by electron microscopy. It produced flagellin proteins with lower apparent molecular masses than wildtype flagellins based on their migration through SDS-PAGE. The mutant FlaB1/FlaB2 proteins (the major flagellins) migrated faster than the corresponding wild type proteins (representing fully modified flagellins) but slower than *M. voltae* FlaB2 protein overexpressed in *Escherichia coli* (representing presumably unmodified flagellin). These data suggest that insertional activation of the lipopolysaccharide GluNAc transferase gene has resulted in an altered pattern of glycosylation on *Methanococcus* flagellins which leads to an inability to produce functional flagella filaments at the cell surface.

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A 2-OXOACID DEHYDROGENASE MULTI-ENZYME COMPLEX IN THE ARCHAEA

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In aerobic bacteria and eukaryotes, a family of 2-oxoacid dehydrogenase multienzyme complexes (OADHCs) are responsible for the oxidative decarboxylation of 2-oxoacids in pathways of central metabolism. These large complexes comprise multiple copies of three enzymes, 2-oxoacid decarboxylase (E1), dihydrolipoyl acyltransferase (E2) and dihydrolipoamide dehydrogenase (E3). E2 forms the complex structural core, with E1 and E3 bound peripherally and non-covalently. No OADHC activity has ever been detected in Archaea; instead, the oxidation of 2-oxoacids is catalysed by much more simple 2-oxoacid ferredoxin oxidoreductases. However, dihydrolipoamide dehydrogenase activity has been detected in a number of Archaeal species, even though the only known function of this enzyme is as the E3 component of an OADHC, or of the glycine cleavage system. Sequencing of the E3 gene from the halophilic Archaeon Haloferax volcanii showed it to possess high sequence identity to E3 of Eukarya and Bacteria, and further sequencing revealed a potential operon encoding E1, E2 and E3 genes, with potential promoter and Shine-Dalgarno sequences located upstream of the first open reading frame. Northern analysis suggested that the operon is transcribed as a single message. More recently, genome sequencing has revealed a similar situation in the genomes of the thermophilic Archaea Thermoplasma acidophilum and Aeropyrum pernix. We will report the successful cloning and expression of all four of the putative OADHC genes of Tp. acidophilum, and purification of the subsequent protein products. The recombinant E1 and E3 enzymes are active, and the 2-oxoacid substrate for this complex has been determined. Complex assembly experiments have commenced, and will also be reported.

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THE THERMOPHILIC BACTERIUM ALICYCLOBACILLUS ACIDOCALDARIUS HAS BETA-GLYCOSIDASE AND BETA-GALACTOSIDASE ACTIVITIES: CLONING AND CHARACTERIZATION OF THE TWO ENZYMES

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The thermoacidophilic bacterium Alicyclobacillus acidocaldarius (formerly Bacillus acidocaldarius) grows aerobically at 60°C and pH 4.0. In cellular extracts of this microorganism we found high b-galactosidase activity and, in a SDS-PAGE after renaturation of the extract in situ, we identified a single band by staining with X-Gal substrate. However, interestingly, by staining with X-Glc a band of higher molecular weight turned out. In an effort to clone and characterize these two enzymes we prepared a genomic library obtained inserting a partial digestion of the A. acidocaldarius genome in the vector Zap-Express that was used to identify clones by functional complementation. To this aim, phagemids produced from the library were used to transform an E. coli strain whose genotype allows the selection of clones on both the chromogenic substrate X-Gal or the disaccharide lactose. The screening of more than 1,000,000 cfu brought to the isolation of clones positive on the X-Gal and able to complement the lack of growth on minimal lactose medium. One of these clones was isolated and completely sequenced: it revealed a complete open reading frame of 1,443 bp encoding for a polypeptide of 480 amino acids that belong to family 1 of glycosyl hydrolases (GH1). The recombinant enzyme, named Aab-gly, is an esamer in native conditions and shows a monomeric molecular weight of about 56,000 Da. Aab-Gly has a specific activity at 70°C of 81 and 84 U/mg on 2-NP-Gal and 2-NP-Glc, respectively, and react with both X-Gal and X-Glc in SDS-PAGE, suggesting broad substrate specificity as the other members of GH1. Surprisingly, all the clones obtained by complementation of the E. coli strain with the A. acidocaldarius library were similar to Aab-gly and even after repeated screenings on two different genomic libraries we could not find clones expressing specific b-galactosidase activities. This suggests that the enzyme, which is expressed at high level in A. acidocaldarius, is not functional in E. coli; therefore, we purified to homogeneity the b-galactosidase

enzyme from A. acidocaldarius. The pure enzyme, corresponding to the high molecular weight band by activity staining on SDS-PAGE, is active exclusively on lactose and aryl-galactosides. The analysis of the N-terminal sequence allowed its classification as a member of GH42 that include only specific b-galactosidases. The cloning and expression of this enzyme are currently in progress.

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ANALYSIS OF THERMAL ADAPTATION IN THE CARBOXYLESTERASE/LIPASE HSL ENZYME FAMILY

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The recently solved 3D structures of two thermostable members of the carboxylesterase/lipase HSL family, namely the Alicyclobacillus (formerly Bacillus) acidocaldarius (1) and Archaeoglobus fulgidus carboxylesterases (2) (EST2 and AFEST respectively) were compared with that of the mesophilic counterpart Brefeldine A esterase from Bacillus subtilis (BFAE) (3). Since the 3D homology models of other members of the HSL family were also available, we performed a structural alignment with all these sequences. The resulting alignment was used to assess the amino acid "traffic rule" in the HSL family. Quite surprisingly, the data were in very good agreement with those recently reported from two independent groups and based on the comparison of a huge number of homologous sequences from the genus Bacillus, Methanococcus and Deinococcus/Thermus. Taken as a whole, the data point to the statistical meaning of defined amino acid conversions going from psychrophilic to hyperthermophilic sequences. We identified and mapped several such changes onto the EST2 structure and observed that such mutations were localized mostly in loops regions or a-helices and were mostly excluded from the active site. A site-directed mutagenesis of two of the identified residues confirmed they were involved in thermal stability (4). These residues were involved in a salt bridge and a network of ion pairs. Therefore we adopted an "alanine-scanning" mutagenic approach in order to measure the contribution to the enzyme stability of residues involved in ion pairs and network of ion pairs that were conserved in EST2 and AFEST but not in BFAE. Fifteen single and five double mutants were produced, the proteins expressed in E. coli purified and characterised kinetically and structurally. Most of mutations were demonstrated to affect enzyme stability as well as kinetic properties. Data will be discussed in the context of the known EST2 3D structure. Experiments of "double-mutant cycle" are currently ongoing in order to dissect the electrostatic from others, concurrent, effects.

1) De Simone G, Galdiero S, Manco G, Lang D, Rossi M, Pedone C. (2000) J Mol Biol. 303,761-71.
2) De Simone G, Menchise V, Manco G, Mandrich L, Sorrentino N, Lang D, Rossi M,Pedone C. (2001) J Mol Biol. 314,507-18.
3) Wei Y, Contreras JA, Sheffield P, Osterlund T, Derewenda U, Kneusel RE, Matern U, Holm C, Derewenda ZS. (1999) Nat Struct Biol. 6, 340-5.
4) Mandrich L, Pezzullo M, Del Vecchio P, Barone G, Rossi M, Manco G. (2004) J Mol Biol. 335, 357-69

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INTRACELLULAR DISULFIDE BOND ABUNDANCE IN HYPERTHERMOPHILIC MICROBES: GENOMIC AND BIOCHEMICAL REVELATIONS

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Recent research has identified a number of hyperthermophilic microbes predicted to contain an abundance of cytosolic protein disulfide bonds [Mallick, *et al.*, (2002)], challenging the long-held view that such bonds are prohibited in the cytoplasm. Here, we provide further experimental data and computational analyses that expand upon these findings and reveal surprising results. 1D and 2D diagonal gel electrophoresis experiments confirm that the hyperthermophilic archaean *Pyrobaculum aerophilum* contains a large fraction of disulfide-bonded intracellular proteins, including a number of protein complexes held together by inter-molecular disulfide bonds. Furthermore, thermal denaturation studies of proteins in cell lysate reveal that the disulfide bonds contribute to protein stability. Though several factors have been identified as potentially contributing to thermal stability in individual proteins, no single characteristic has been found to be a major stabilizing factor of thermophilic proteins in general. Our findings suggest that, in at least some hyperthermophilic organisms, disulfide-bonds constitute a single major contributing factor to the structural stability of a significant fraction of cytosolic proteins. Various related computational studies are also underway. These studies aim to (1) illuminate from a bioinformatics perspective how these cells can maintain intracellular disulfide bonds, (2) develop special amino acid substitution tables for disulfide rich organisms, and (3) improve protein fold prediction methods on the basis of disulfide bond information.

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ALKALINE PROTEASES PURIFIED FROM AN ALKALINE *BACILLUS* STRAIN ISOLATED AND CHARACTERIZED FROM A SODA LAKE IN TANZANIA

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An alkaliphilic bacterium, designated strain A11, was isolated from a Tanzania soda lake in Africa. The organism was characterized to be of rod shape, gram positive, motile, flagellated, obligate aerobic and alkaliphilic with an optimal growth rate at pH 10. The strain A11 can tolerate up to 15% (w/v) NaCl and grow on a wide range of carbon sources between temperatures 18C-45C. Sequence analysis of the 16S rRNA gene revealed that the isolate was associated with the members of the genus *Bacillus* with a relationship of 96 % identity to *B. agaradhaerens*. These two organisms show significant differences in GC content, DNA-DNA hybridization, physiological properties and cellular fatty acid composition between each other such that A11 is proposed to be a novel alkaliphilic species of the

Bacillus genus. Two forms of extracellular metalloproteases have been produced from A11 with molecular sizes of 25 kDa and 30kDa. The proteases have been purified to homogeneity, based on SDS-PAGE analysis, from the liquid culture of this organism, and their biochemical properties characterized. The maximum enzymatic activities against azoalbumin for the two proteases were observed at 45C and at 50C for the 25kDa and 30kDa proteins respectively at pH 9.5. We describe the enzymatic properties of these extracellular proteolytic enzymes and compare them to other known alkaliphilic proteases.

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ASPARTATE TRANSCARBAMYLASE OF THE HYPERTHERMOPHILIC ARCHAEON PYROCOCCUS ABYSSI: STRUCTURE OF THE CATALYTIC SUBUNIT, THERMOSTABILITY AND INSIGHTS INTO THE ALLOSTERIC MECHANISMS

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Aspartate transcarbamylase activity is present in all living organisms, under a large variety of molecular organizations. The P. abyssi ATCase is a dodecamer comprising two trimeric catalytic subunits associated with three regulatory subunits, like Class B bacterial ATCases. The isolated catalytic subunit is intrinsically thermostable but its association with regulatory subunits to form the holoenzyme was found to increase thermostability significantly. The P. abyssi ATCase is cooperative toward the substrate aspartate and is allosterically regulated by nucleotides : CTP and UTP are inhibitors and ATP is an activator. Cooperativity was found to result from a transition from a low affinity T state to a higher affinity R state, induced by aspartate or by analogues. Sedimentation velocity experiments showed that this transition is characterized by a significant conformational change. Competition binding experiments showed that the allosteric effectors bind competitively to the same sites on the regulatory subunits. The structure of the isolated subunit, liganded with the bisubstrate analogue N-phosphonacetyl-L-aspartate, was solved to 1.8 Å resolution. A comparison with the E. coli catalytic subunit shows that the active site is highly conserved. However, a loop contributing two residues to the catalytic site is shorter by one residue than in the E. coli ATCase. Although all known thermophilic and hyperthermophilic ATCases show this shorter loop, it is also found in several mesophilic ATCases. It is proposed that the shorter loop is related to the channeling of the highly thermolabile second substrate of the enzyme - carbamyl phosphate - between carbamyl phosphate synthase and ATCase rather than to the adaptation to high temperature.

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ENZYMATIC PROPERTIES, CATALYTIC RESIDUES, AND X-RAY STRUCTURE OF ALPHA-1,4-GLUCOSIDASE FROM GEOBACILLUS SP. STRAIN HTA-462

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alpha-1,4-glucosidase from Geobacillus sp. strain HTA-462, one of the deepest sea bacteria isolated from the sediment of the Mariana Trench, was purified to homogeneity and its enzymatic and structural characteristics have been studied. The enzyme was most stable at pH 9.0 and possessed maximum activity at 60oC and at pH 9.0. Interestingly, the enzyme possessed an extraordinarily high transglycosylation activity and could utilize various non-sugar molecules as sugar acceptors. In certain conditions, the enzyme also converted maltose to isomaltose. The gene encoding the enzyme was cloned and sequenced. The recombinant enzyme could be secreted by Bacillus subtilis harboring its gene and preserved primary properties of the native enzyme. The native enzyme was successfully crystallized and its X-ray diffraction data to 2.5 Å resolution were collected and processed. The overall structure and active site of the enzyme revealed from X-ray structural and site-directed mutagenesis studies will be presented and discussed.

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PATHWAY OF IRON OXIDATION IN AN ACIDOPHILIC BACTERIUM, ACIDITHIOBACILLUS FERROOXIDANS: STRUCTURAL AND METABOLIC STUDIES

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Acidithiobacillus ferrooxidans is an acidophilic chemolithoautotrophic gram-negative bacterium that derives energy from the oxidation of ferrous iron, elemental sulfur and various sulfur compounds at pH 2 using oxygen as the electron acceptor. The study of this bacterium presents economic and fundamental biological interests. It oxidizes metal sulfides like pyrite enabling solubilization and valorization of precious metals. In addition, this extreme acidophilic bacterium lives at pH values below 2 and implies special structure-function relationships. The bioenergetic metabolism of the ore-leaching proteobacterium *A. ferrooxidans* involves an electron transfer chain coupling oxidation of iron (II) at the external cell wall surface to reduction of the terminal electron acceptor, molecular oxygen. Most of the metalloproteins involved in the periplasmic respiratory chain are acid-stable proteins exhibiting very high redox potentials. We have isolated and characterized two dihemic c4-type cytochromes, cytochrome c4 [21000] (CYC41) and cytochrome c4 [26000] (CYC42). These cytochromes present 34.5% amino acid identity, differ by molecular mass, EPR spectra, and redox potentials. Both cytochromes interact with a blue copper protein: the rusticyanin (Rcy). *A. ferrooxidans* is the only organism reported so far that

harbours two different representatives of this diheme cytochromes family. This raises the question of the structural and functional characteristics favoring the presence of c4-type diheme over the use of standard monoheme cytochromes. We have resolved the CYC_{41} 3D structure which reveals that the overall fold corresponds to that of a typical neutrophilic c_4 cytochrome. However differences are observed in the repartition of type bonds found in the structure stabilization. CYC_{41} is involved in electron transfer between Rcy and the cytochrome oxidase an inner membrane protein which is the physiological terminal acceptor. In order to resolved the electron flow pathway between this cytochrome and its two partners, we have performed site-directed mutagenesis of CYC_{41}. We have found physiological conditions to product this acidophilic cytochrome in *E. coli* which reveals crucial role of residues ionization state in the structural stabilization of the protein. We proposed a model where CYC_{41} function like electron wire using one heme as the entrance accepting electrons from the Rcy and the other heme as the exit giving electrons to the cytochrome oxidase.

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REGULATION OF SALT-TOLERANT GLUTAMINASE FROM *MICROCOCCUS LUTEUS* K-3 BY SODIUM CHLORIDE AND ADENINE NUCLEOTIDES

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L-glutaminase catalyses the hydrolysis of L-glutamine to L-glutamic acid and ammonia and has a central role in cellular nitrogen metabolism. In recent years glutaminase attracted much attention with respect to its proposed applications in both pharmaceuticals and food industries. Salt-tolerant and thermo-tolerant glutaminases have drawn considerable interest as flavor enhancing additives in food fermentations especially in soy sauce fermentation. Glutaminase from *Micrococcus luteus* K-3, an isolate from Beppu bay in Japan, and overexpressed in *E.coli* was stable even in the presence of 3.1M NaCl. High concentrations of NaCl not only imparted thermal stability but also enhanced the catalytic activity demonstrating its salt tolerance. Adenine nucleotide, ADP and ATP inhibited the enzyme significantly, but was reversible by the presence of NaCl. AMP did not affect the enzyme activity however, in the presence of NaCl, AMP resulted in 50% inhibition of the optimal enzyme activity. It has been observed that with increased NaCl concentration, the enzyme structure gradually became susceptible to be modified by diethylpyrocarbonate. This change of structure was found to be slight as there is no remarkable change in the CD spectra.

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ENZYME CHARACTERISTICS OF FARNESYL DIPHOSPHATE/GERANYLGERANYL DIPHOSPHATE SYNTHASE FROM HYPERTHERMOPHILIC ARCHAEON, *THERMOCOCCUS KODAKARAENSIS*

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One of the most characteristic features of archaea is the molecular architecture of their membrane lipids, which seems to permit their survival under severe conditions, e.g., at high temperatures or extreme pHs. Archaeal core membrane lipids (archaeols) consist of diethers containing two C_{20} isopranoid (phytanyl) chains attached to glycerol. The *Tk-idsA* gene is an ortholog of trans-prenyl diphosphate synthases from hyperthermophilic archaeon *Thermococcus kodakaraensis*, which catalyze the consecutive trans-condensation of isopentenyl diphosphate (C_5) units with allylic diphosphate. Recombinant form of *Tk*-IdsA was obtained and the biochemical characteristics were examined. Thermodynamic analyses were performed by the circular dichroism monitoring, showing that maximum free-energy change ($\Delta G_{0\,max}$), melting temperature (*Tm*), change of enthalpy (ΔHm) and heat capacity change (ΔCp) of *Tk*-IdsA were calculated as 16 kJ mol^{-1} at 60°C, 91.0 °C, 307.6 kJ $mol^{-1}$and 8.5 kJ mol^{-1} K^{-1}, respectively, indicating that *Tk*-IdsA is the most thermostable trans-prenyl diphosphate synthases among known counterparts. Product analysis revealed that *Tk*-IdsA is a bifunctional enzyme, farnesyl diphosphate (FPP, C_{15})/geranylgeranyl diphosphate (GGPP, C_{20}) synthase, which mainly yields both C_{15} and C_{20}. It is thought that these products are used for the biosynthesis of the membrane components. Further investigations performed at 70°C and 90°C showed that the FPP/GGPP product ratio increases with the rise of the reaction temperature. The kinetic parameters obtained at 70°C and 90°C also demonstrated that the rise of the temperature elevates the k_0 value for the C_{10} allylic substrate more dramatically than those for the C_5 and C_{15} allylic substrates. These data suggest that *Tk*-IdsA contributes to adjust the membrane composition to the cell growth temperature by modulating its substrate and product specificities. Moreover, single-mutated enzyme *Tk*-IdsA-Y81S, in which Tyr-81 was replaced with Ser, gave C_{25} and C_{30} products, indicating that bulky side-chain at this position inhibits condensation of IPP beyond the C_{20} and Tyr-81 is crucial for defining the final product length as C_{20}. Another mutant *Tk*-IdsA-Y81A, in which Tyr-81 was replaced with Ala, produced further longer chain product (>C_{50}). These results suggest that an elimination of the bulky side-chain and elevating hydrophylicity at the position allows the enzyme to produce longer chain product.

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WRBA FROM *ARCHAEOGLOBUS FULGIDUS* IS A NAD(P)H: BENZOQUINONE OXIDOREDUCTASE THAT COULD FUNCTION IN A STRESS-RESPONSE

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The tryptophan (W) repressor-binding protein (WrbA) was first discovered in *Escherichia coli* and was proposed to function in concert with the Trp repressor, although this hypothesis was not investigated. WrbA has been described as a flavodoxin-like protein, but the protein contains several unique sequence inserts that, along with a loosely-bound FMN cofactor and multimerization, make this a distinct family of proteins with *E. coli* WrbA being the founding member. Several global expression studies demonstrate that *E. coli wrbA* is under the control of RpoS (the stress-response sigma factor, ó^{s} or ó^{38}) and is upregulated in response to stressors such as acids, salts, hydrogen peroxide, and diauxie, as well as in the early stages of the stationary phase. Sequence similarity to NADH: benzoquinone oxidoreductases (NBOs) suggests that WrbA may reduce quinones, possibly functioning at the membrane to sense or stabilize the redox state of the cell in response to environmental stress. In the genome of *Archaeoglobus fulgidus*, *wrbA* is flanked by neelaredoxin (*nlr*) and a putative nigerythrin (*ngr*), two iron-containing proteins involved in alleviating oxidative stress. I have heterologously expressed and purified the WrbA homologue from *A. fulgidus* and have shown that this WrbA homologue can be reduced by NADH and NADPH and can reduce benzoquinone and potassium ferricyanide. The activity reported for the WrbA homologue from *A. fulgidus* is consistent with two potential roles: WrbA could function as a NBO to signal cellular stress or to alleviate oxidative stress, and WrbA could also function as a $1e^{-}/2e^{-}$ switch, coupling electron transfer from NAD(P)H to proteins that reduce reactive oxygen species.

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ACTIVITY, STABILITY AND FLEXIBILITY OF A COLD-ACTIVE AMINOPEPTIDASE PRODUCED BY MARINE PSYCHROPHILE COLWELLIA PSYCHRERYTHRAEA STRAIN 34H

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Microorganisms in perennially-cold (near 0°C) marine environments play important roles in the degradation of organic matter via the activity of their extracellular enzymes even in the coldest of habitats (sea ice). Despite their environmental and physiological significance, mechanisms allowing enzymatic activity in the cold are not completely understood. In this study we successfully overexpressed an extracellular M1 aminopeptidase produced by the Arctic marine psychrophile Colwellia psychrerythraea strain 34H at 18°C in E. coli BL21 (DE3). The recombinant enzyme was purified to homogeneity using anion exchange and hydroxyapatite chromatographic techniques. Here the structural, biochemical and biophysical characteristics of the cold-active aminopeptidase (ColAP) will be compared with those of mesophilic homolog, Leukotriene A4 hydrolase, a bifunctional enzyme from Homo sapiens. Ongoing characterization of ColAP is designed to investigate the relationship between enzyme activity, flexibility and stability thus helping to elucidate specific mechanisms enabling maintenance of enzyme function at low temperatures.

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IDENTIFICATION, CLONING AND EXPRESSION OF THE SULFITE OXIDASE GENE FROM *DEINOCOCCUS RADIODURANS* R1

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Sulfite plays a key role in oxidative sulfur metabolism as the main intermediate in the oxidation of sulfur compounds to sulfate; its nucleophilicity and strong reducing capability contribute to its toxicity and antimicrobial action which have led to its widespread use as a food preservative. Direct oxidation to sulfate catalyzed by sulfite:acceptor oxidoreductases appears to have the wider distribution; nevertheless, in general the information available on bacterial sulfite-oxidizing enzymes reveals great diversity, e.g. with respect to cellular localization, molecular mass and catalytic parameters. In many pro- and also eukaryotes sulfite is formed as a degradative product from molecules containing sulfur as a heteroatom. In these organisms detoxification of sulfite is generally achieved by direct oxidation, in which sulfite oxidases (SOX) catalyse the two electron oxidation of sulfite to sulphate ($SO_3^{2-} + H_2O$! $SO_4^{2-} + 2H^+ + 2\ e^-$). SOXs are ubiquitous among different sources and in all known cases, these enzymes possess a molybdenum centre (Moco: Molybdenum cofactor) consisting of a single cofactor dithiolene coordinated to *cis* MoO_2 unit; the additional coordination position may be taken up by a cystein residue that is conserved within the family. *Deinococcus radiodurans* is a poly-extremophile bacterium, first identified in 1956 as surprising contaminant of heavily irradiated canned food. It survives in a variety of environments of radioactivity and ultraviolet radiation, genotoxic chemicals, heat, desiccation and severe acceleration and deceleration forces which are lethal to almost all other cellular species. The complete sequence of the bacterium genome is available: it comprises two circular chromosomes and two plasmids, with between 4 and 10 copies of the complete genome. The intrinsic stability of its enzymes and their resistance to denaturing physical and chemical factors are considerable advantages in industrial processes and in biotechnological applications. On the chromosome II an open reading frame (DRA0225) was identified as a putative sulfite oxidase gene; the translated protein shows high aminoacid sequence similarity to eukaryotic and prokaryotic sulfite oxidases. The sequence was PCR amplified from genomic DNA of *Deinococcus radiodurans* R1 and heterologous gene expression obtained as a 6xHis-tagged protein in *E. coli*, under condition suitable to reduce the formation of inclusion bodies. The recombinant protein was purified by Ni-chelating affinity chromatography and showed an apparent molecu-

lar mass of 39 kDa, as expected for the translated gene sequence. The predicted enzyme activity was confirmed by specific assays using the potassium ferricyanide or cytocrome C as the electron acceptors.

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ANALYSIS OF GENES RESPONSIBLE FOR OLIGO- TO MONO-SACCHARIDE METABOLIC CONVERSION IN *SULFOLOBUS SOLFATARICUS*

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Cellulose and hemicellulose are the major polymeric component of plant matter and the most abundant polysaccharides on earth. In a typical cellulose-degrading ecosystem, a variety of cellulolytic bacteria and fungi works in concert with related microorganisms to convert insoluble complex polymeric substrates to soluble sugars. In order to catalyse this process, the cellulolytic microbes produce a variety of different enzymes, known as cellulases, hemicellulases and xylanases. The reaction that they catalyse is the hydrolysis of the â-glycosidic bonds in the polymeric sugar backbone. Among Crenarchaea, *Sulfolobus solfataricus* (optimal growth at 80°C and pH 2-4), is the most widely studied organism for research on metabolic degradation of complex polysaccharides. Extracellular free and cell-associated cellulase and xylanase activities of this organism have been already identified (1,2) and are currently under study. Furthermore, conversion of oligo- and disaccharide to monosaccharides have been assigned to cytoplasmic glycoside hydrolases, including á-fucosidase (Sso11867/Sso3060), á-xylosidase (Sso3022) and â-glucuronidase (Sso3036). In this respect we identified three ORFs: Sso3032 showed significant similarity to bacterial bifunctional â-xylosidase/á-arabinofuranosidase activity; Sso1353 and Sso1948 were suspected to possess â-glucosidase activity, although no close homolog could be found in the data bank. Sso3032 gene was expressed in *E. coli* and the gene product definitively identified for its substrate specificity. Interestingly, the enzyme showed high efficiency in the conversion of xylo-oligosaccharides, generated by enzymatic xylan degradation, to xylose and was completely inactive against intact polymers. Both Sso1353 and Sso1948 genes lie downstream of two putative cellulase genes (Sso1354 and Sso1949). The protein lenght is 663aa for Sso1353 and 661aa for Sso1948 and there is no clear signal peptide at their N terminal. Moreover, the homology between the two proteins gives an identity score of 86%. The vicinity of these genes to the putative cellulases in the *S. solfataricus* genome strongly suggested their involvement in cellulose degradation. In order to verify this hypothesis, we cloned their complete coding sequence and expressed in the vector pGEX2TK in *E.coli*. The proteins were purified and assayed for specificity by the Somogji-Nelson method with xylans, xyloglucan, xyloglucan oligosaccharides, cellotriose, cellotetraose, cellopentaose, cellobiose, and lactose as substrates. The putative function was confirmed since only with xyloglucan oligosaccharides and with the short cellulose oligomers, appreciable amount of reduced sugar were obtained.

1. Limauro D., et al. (2001) *Extremophiles* **5**, 213-9.
2. Cannio R., et al. (2004) *Extremophiles* **8**,117-24.

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INVESTIGATION OF FACTORS INVOLVED IN THERMOSTABILITY OF ISOCITRATE DEHYDROGENASE (IDH) FROM THE HYPERTHERMOPHILIC ARCHAEON *AEROPYRUM PERNIX* (*Ap*IDH)

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*Ap*IDH has been crystallized and the three-dimensional structure solved (1). A comparative structural study of *Ap*IDH with IDH from the mesophilic bacterium *Escherichia coli*, has shown that ion-pair networks and a disulphide-bond at the N-terminal are possible determinants for thermostabilty. Introduction of the mutation Cys87Ser with the aim of disrupting the disulphide bonding, resulted in an enzyme with an apparent melting temperature of 100.3 ºC i.e. 9.6 ºC less then the native recombinant enzyme (109.9ºC) (2). This result confirms the N-terminal disulphide bridge as important for thermostabilization of *Ap*IDH. Furthermore, the introduction of mutations; D130N, D344N, R211Q and R211M with the aim of disrupting a 7-member ion-network, resulted in 4 mutants with lower apparent melting temperatures then the native recombinant enzyme. R211M had the most considerable effect on stability of the enzyme with an apparent melting temperature of 98.6ºC, i.e 11.3ºC less then the native recombinant enzyme. An ion-pair was also detected at the subunit interface of *Ap*IDH and point mutations were introduced with the aim of disrupting this connection. In *E.coli* IDH, which has three ion-pairs at the subunit interface, it is believed that these ion-pairs are involved in subunit assembly. Mutations at the subunit interface of *Ap*IDH resulted in mutants; E188Q and E188A, with melting temperatures close to the native enzyme; 108.5ºC and 107.6ºC, respectively, and therefore it is assumed that they have an insignificant contribution to the thermostabilization of *Ap*IDH.

1. Karlström, M et al. (2004), manuscript in preparation.
2. Steen, I.H, Madern, D., Karlström, M., Lien, T., Ladenstein, R. and Birkeland, N-K (2001). *The Journal Of Biological Chemistry*. 276, 43924-43931

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COMPARATIVE PH STUDY ON L-ARABINOSE ISOMERASES FROM *BACILLUS HALODURANS, GEOBACILLUS STEAROTHERMOPHILUS* AND *ALICYCLOBACILLUS ACIDOCALDARIUS*

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Three *araA* genes encoding L-arabinose isomerase (L-AI) from *Bacillus halodurans*, *B. stearothermophilus*, *Alicyclobacillus acidocaldarius* were cloned and overexpressed in *E. coli*. The recombinant enzymes

were purified to homogeneity by heat treatment Q-sepharose fast flow ion exchange chromatography and gel filtration. Each native enzyme was estimated by native PAGE to be a homotetramer with a molecular mass of about 230 kDa. Although these enzymes originated from microorganisms that have different growth pHs, they exhibit high levels of amino acids sequence similarity (>83%). Especially, only eleven aimino acid differences were detected between *G. stearothermophilus* and *A. acidocaldarius* AIs. The differences of their biochemical properties are very interesting in view of their close similarity in primary and secondary structures. For industrial applications, an increase pH stability of AI in the isomerization D-tagatose from D-galactose should reduce by-products for commercial production of D-tagatose as a low calorie bulk sweetener. Therefore, these enzymes may be a good model system for the analysis of pH stability and for the production of D-tagatose as a novel sweetener. The present study was undertaken to compare the biological and physicochemical properties of L-AIs from neutrophilic, alkalophilic, and acidophilic microorganisms with respect to their pH dependant optimum and stabilities based on pH-dynamics by using of site-directed mutagenesis and CD spectropolarimetry.

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STRUCTURE-FUNCTION RELATIONSHIPS OF A CATALASE FROM *EXIGUOBACTERIUM OXIDOTOLERANS* SP. NOV. SURVIVING HIGH CONCENTRATION OF H_2O_2

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Extracellular and/or intracellular catalase(s) protects the organisms against oxidative damage to eliminate H_2O_2. A novel bacterium exhibiting high H_2O_2 resistance and high catalase activity without induction of oxidative stress has been isolated from a water sample collected from a drain pool of a fish-processing plant using H_2O_2 as a bleaching agent in Hokkaido. The isolate resists 100 mM H_2O_2 and does not incur severe damage even at 500 mM H_2O_2, whereas *Escherichia coli* exhibits sensitivity higher than 10 mM H_2O_2. Its cell extract shows 558 times higher catalase activity than that of *E. coli*. On the basis of phenotypic characteristics, phylogenetic position (as determined by 16S rRNA gene sequence analysis) and DNA-DNA relatedness, the isolate has been identified as a novel species, for which the name *Exiguobacterium oxidotolerans* sp. nov. has been proposed. The catalase (EKTA) of *Exiguobacterium oxidotolerans* was purified 23-fold from cell extract, and exhibited a specific activity of 430,000 U•mg of protein^{-1} at 25 °C in 50 mM sodium phosphate buffer (pH 7.0) containing 30 mM H_2O_2. Absorption spectrum of EKTA showed a Soret band at 408 nm with broad peaks at 506 nm and 627 nm suggested that is a typical monofunctional catalase containing Fe(III)-protoporphyrin IX. The formation rate of the reactive intermediate of the catalase reaction was demonstrated on a stopped-flow apparatus using peracetic acid. The observed reaction rates were at least of 1 order and 2 orders magnitude faster than catalase of bovine liver and *Micrococcus luteus*, respectively. The fact suggests that the substrate accessible mechanism in EKTA is different from other catalases. The gene encoding EKTA was obtained by inverse PCR method and the DNA sequence was determined by direct sequencing. Based on the DNA sequence, EKTA was comprised of 491 amino acids and the calculated molecular weight was 56,523 Da. The X-ray crystal structure of native EKTA revealed that the substrate-access channel comprised of unique hydrophobic amino acid residues. This result implicates that the access of H_2O_2 and organic peroxides to the active site would be accelerated by the hydrophobic nature of the substrate-access channel.

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TOWARDS METAL-FILTERED FUNCTIONAL PROTEOMICS; DETECTING AND CHARACTERIZING METAL CONTAINING PROTEINS BY *NATIVE-NATIVE* 2D-GE

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It is a key challenge for the 21th century to make sense of translated genome information at the molecular level. Our approach is metal-filtered proteomics: in order to reduce the multidimensionality of whole cell proteomics it is necessary to filter down the number of gene products to a tractable set while keeping main aspects of interrelationship between the gene products. It is assumed that all proteins associated with a particular metal are significantly interrelated through metal homeostasis. In this research the metal tungsten was used as a filter to identify related proteins of *Pyrococcus furiosus*. Proteins will usually lose their metal cofactors in SDS and urea; SDS-PAGE and standard 2D-GE cannot be used to detect these metal proteins and, therefore *native-native* 2D gel electrophoresis is developed. The technique of separation is conventional: in the first dimension it is based on differences in charge and in the second dimension on differences in size. In order to be able to detect these proteins radioactively labeled tungsten (^{187}W, $t_{0.5}$=24 h) was added to the growth media. 2D gels were made with cell free extract and they were read out using a phosphor screen instead of autoradiography, for increased sensitivity. Four tungstozymes have previously been purified to homogeneity from *P. furiosus*, and three more putative W-proteins are identified by comparative genome annotation. With the *native-native* method already more spots can be found on the gel, and with higher resolution the number is expected to increase further. When the radioactivity has decayed the protein spots can be cut out and, e.g., identified by MALDI-TOF mass spectrometry. When the *native-native* method is compared to other detection methods like coomassie blue, silver and other staining solutions, the dynamic range over which proteins can be detected does not depend on amount of protein but on amount of specific radioactivity (Bq/mg). This can easily be varied by adding tungsten with a higher specific radioactivity. Also, the sensitivity of the method is higher compared to other methods to detect metals in proteins as, for example electrochemical determinations with mercury electrodes or inductively coupled plasma emission spectrometry. Furthermore, this method can be used on many different cells and several target metals can be used depending on the lifetime and the specific activity of the isotopes.

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ALKALI-INDUCIBLE BACTERIAL FLOTILLIN-LIKE PROTEIN FROM BACILLUS HALODURANS C-125

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Flotillins are the marker for lipid microdomains emerged as a key concept in cell biology. Although the potential homologues were detected in bacteria through Blast search analysis, the characteristic of bacterial flotillin-like proteins has not been investigated. Amino acid sequence of the BH3500 protein from Bacillus halodurans, was approx. 53% similar to that of flotillin-1. Motif analysis revealed that several specific residues (SPFH-, FLOTILLIN- domain, AEA-repeat structure) and 5 potential phosphorylation sites for casein kinase II, tyrosine kinases and protein kinase C are conserved in the BH3500 protein. In addition, the BH3500 protein possessed two transmembrane-spanning domains at N-terminus that is consistent with common properties of flotillin-1. The BH3500 protein was detected in Triton-insoluble, buoyant membrane fraction of B. halodurans by mass spectrometry and Western Blotting. Interestingly, BH3500 was expressed strongly in alkaline conditions at both transcriptional and translational levels, implying that it is one of alkali-inducible proteins.

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A STRUCTURE-SPECIFIC DNA SUBSTRATE INDUCES CONFORMATIONAL CHANGES IN TAQ POLYMERASE

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The active sites of the polymerase and 5'-nuclease domains of DNA polymerase I from *Thermus aquaticus* (Taq polymerase) are about 70 Å apart in the crystal structure. It is unclear how Taq polymerase coordinates its polymerase and the nuclease activities to generate only a nick, rather than a gap or an overhang, on a DNA duplex during lagging-strand DNA synthesis and DNA repair. Using contrast variation solution small angle neutron scattering (SANS), we have examined the conformational changes that occur in Taq polymerase upon binding "overlap flap" DNA, a structure-specific DNA substrate that mimics the substrate in strand replacement reactions. In solution, apo Taq polymerase has an overall expanded equilibrium conformation similar to that in the crystal structure. Upon binding to the DNA substrate, both polymerase and nuclease domains adopt more compact overall conformations, but these changes appear to be not enough to bring the two active sites into a proximity close enough to generate a nick. Reconstruction of a 3-D molecular envelope from SANS data shows that in the DNA-bound form, the nuclease domain is lifted relative its position in the non-DNA-bound form so as to be in closer contact with the thumb and palm of the polymerase domain. The results suggest that different preferences of the polymerase and nuclease domains for the growing upstream stand and the overlap flap substrate, respectively, are responsible for coordination of polymerase and nuclease activities during nick generation. Nevertheless, it remains likely that direct interaction between polymerase and nuclease domains assists in the transfer of DNA substrate from one active site to the other.

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OXYGEN SENSITIVITY OF 2-KETOACID FERREDOXIN OXIDOREDUCTASES FROM A HYPERTHERMOPHILIC ARCHAEON *THERMOCOCCUS PROFUNDUS*

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A hyperthermophilic archeon *Thermococcus profundus* contains four types of 2-ketoacid ferredoxin oxidoreductase: pyruvate ferredoxin oxidoreductase, PFOR; 2-ketoisovalerate ferredoxin oxidoreductase, VOR; 2-ketoglutarate ferredoxin oxidoreductase, KGOR; indolepyruvate ferredoxin oxidoreductase, IOR. These enzymes catalyze oxidative decarboxylation reactions of many 2-ketoacids that are produced from amino acids. The former three, PFOR, VOR, KGOR, are octamer which are composed from four types of subunits, while IOR is a heterotetramer that is composed from two types of subunits. They contain, in commom, thiamine pyrophosphate (TPP), magnesium ion, 3Fe-4S and/or 4Fe-4S clusters. PFOR contains copper ion additionally. In this study we investigated oxygen sensitivity of these four enzymes, mainly of VOR. The half-life of *T. profundus* VOR was 4 min at 25°C under aerobic conditions. Therefore purifications have been done under extremely anaerobic conditions. Under such conditions, we obtained purified VOR preparations whose specific activities were varied (20 - 910 units/mg). With two spectroscopies (EPR and resonance Raman), we obtained data which suggest that the enzyme preparations with high specific activities contains larger amounts of 4Fe clusters. In addition, the content of the 4Fe cluster were decreased when the enzyme were exposed to air with concomitant loss of the enzyme activities. These results indicate that the 4Fe cluster(s) are important to exhibits enzyme activity and also suggest that VOR contains 4Fe clusters alone *in vivo*. We will compare these data with those of oxygen sensitivities obtained with other 2-ketoacid ferredoxin oxidoreductases.

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PROTEIN DISULFIDE OXIDOREDUCTASES FROM THE ARCHAEA *PYROCOCCUS FURIOSUS, AEROPYRUM PERNIX* AND THE BACTERIUM *AQUIFEX AEOLICUS*: MEMBERS OF A NOVEL PROTEIN FAMILY IN EXTREMOPHILES RELATED TO PROTEIN DISULFIDE-ISOMERASE (PDI)

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A new family of protein disulfide oxidoreductases with two CXXC active site motifs, characteristic of extremophiles (like DsbA in Bacteria and PDI in Eukarya) has been identified. Protein disulfide oxidoreductases are redox enzymes that catalyse dithiol-disulfide exchange reactions in eukaryotic and bacterial cells. A highly thermostable protein disulfide oxidoreductase was isolated from *Pyrococcus furiosus* (1) and structural details suggested it may be related to the multidomain eukaryotic PDI (*Pf*PDO) (2,3,4). *Pf*PDO possesses a sequence CQYC at the N-terminal active site and at the C-terminal the typical sequence CPYC, which is also found in most glutaredoxins. Functional data supported the hypothesis that *Pf*PDO is an ancestor of the eukaryotic PDI (5). The genomes of archaea and bacteria showed hypothetical thioredoxins and glutaredoxins similar to *Pf*PDO with two active sites. Two putative members of this family have been also identified in the bacterium *Aquifex aeolicus* (*Aa*PDO) and in the archaeon *Aeropyrum pernix* (*Ap*PDO). These proteins resulted to be natural interesting mutants of *Pf*PDO because *Ap*PDO has a different active site at N-terminus CETC and an identical site at C-terminus respect to *Pf*PDO, while *Aa*PDO has two different sequences at the active sites, CESC and CGYC respectively. In order to characterize the new family PDO adapted to extreme conditions, *Ap*PDO and *Aa*PDO have been expressed in *E. coli* and purified to homogeneity. Both the proteins resulted to be fully active in reductive, oxidative and isomerase assays and *Ap*PDO proved to be always the less active. In addition, modelling of *Aa*PDO and *Ap*PDO have been performed using as template *Pf*PDO structure. These models confirm the similarity of the structures. The conclusion between structure and function of the active sites will be described in detail. In addition from comparative genomics and functional genomic studies we have confirmed the function of these proteins and the belonging to this new family.

1. Guagliardi A., de Pascale D., Cannio R., Nobile V., Bartolucci S., Rossi M. 1995. *J. Biol. Chem.* 270: 5748-5755
2. Ren, B., Tibbelin, G., De Pascale, D., Rossi, M., Bartolucci, S., and Ladenstein, R. 1997 *J. Struct. Biol.* 119, 1-5
3. Ren B., Tibbelin G., de Pascale D., Rossi M., Bartolucci S., Ladestein R. 1998. *Nat. Struc. Biol.* 5: 602-611
4. Bartolucci S., de Pascale D., Rossi M. 2001. *Methods Enzymol.* 334: 62-73
5. Pedone E, Ren B., Ladestein R., Bartolucci S., 2004, submitted

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THE DETERMINATION OF CRITICAL SIGNAL PEPTIDE LENGTH FOR PREFLAGELLIN PEPTIDASE PROCESSING IN ARCHAEA

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In Archaea, the preflagellin peptidase (FlaK in *Methanococcus voltae*) is a dedicated enzyme responsible for cleaving the unusual N-terminal signal peptide from preflagellins and in some cases a limited number of other substrates such as sugar-binding proteins. In methanogens and several other Archaeal species, the typical flagellin signal peptide is 11-12 amino acids in length. However, some other predicted substrates of preflagellin peptidase contain unusual, often extremely short, signal peptides. These unusual signal peptides are deduced exclusively from complete genome sequences with no biochemical or genetic verification. To address the relationship between signal peptide length and proper processing by preflagellin peptidase, *Methanococcus voltae* preflagellin FlaB2 with signal peptides 6-12 amino acids in length were generated. These were used as substrate in an in vitro preflagellin peptidase assay utilizing *M. voltae* membranes as an enzyme source, with the processed and unprocessed forms of the flagellins detected by western blotting using anti-flagellin antisera. Significant processing was observed in FlaB2 proteins containing signal peptide shortened to 10 amino acids, whereas mutants with signal peptides at or below 9 amino acids in length were not processed. Thus, besides the conserved amino acids surrounding the cut site, it appears that the length of the signal peptides is also crucial for proper processing. Organisms with preflagellins predicted to possess signal peptides well below the critical length needed in *M. voltae* for proper processing include *Pyrococcus abyssi*, *Pyrococcus furiosus*, *Pyrococcus horikoshii* and *Aeropyrum pernix,* with predicted signal peptides of only 4-6 amino acids. The results presented here would suggest either that these organisms possess preflagellin peptidases with the capacity to process these much shorter signal peptides, or that the current annotation of these genes has resulted in an incorrect translation start site. Biochemical work remains to be done to accurately identify the true translation start sites in these sequences. This is the first report to systematically address the signal peptide length requirement for preflagellin processing. Further studies could answer the interesting question of whether the length of the signal peptide is conserved among all the preflagellin peptidase substrates in the Archaea.

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AN NADP-SPECIFIC ALCOHOL DEHYDROGENASE FROM A HYPERTHERMOPHILIC BACTERIUM THERMOTOGA HYPOGEA

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Thermotoga hypogea is an anaerobic hyperthermophilic bacterium able to grow at 90°C. It utilizes carbohydrates and peptides as carbon and energy sources, and produces acetate, CO2 and hydrogen as major end products. Hydrogen sulfide is generated if

thiosulfate is present in the growth media. In addition, alcohol is also produced as an end product of the fermentation. It is intriguing to investigate what the metabolic pathway of alcohol production is in T. hypogea. There must be two types of enzymes functioning in the pathway. One of them catalyzes the formation of aldehyde, which may be a pyruvate decarboxylase or a two-enzyme system catalyzing the formation of acetyl-CoA that is then reduced to acetaldehyde. However, no CoA-dependent aldehyde dehydrogenase activity has been detected. It is likely that pyruvate decarboxylase is responsible for the formation of acetaldehyde from pyruvate, an intermediate metabolite of central metabolic pathways. The second one, alcohol dehydrogenase, catalyzes the formation of alcohol from the acetaldehyde. Here, we report that an alcohol dehydrogenase is found to be present in the cytoplasmic fraction of the cell of T. hypogea. Its expression appears independent of growth phases. The alcohol dehydrogenase has been purified to homogeneity using an FPLC system. The purified enzyme is a homodimer of a single subunit of 40 kDa revealed by SDS-PAGE. It is oxygen-sensitive, and its activity increases along with the rise of temperature (up to 95°C). Partial sequence analysis shows similarity to iron-dependent alcohol dehydrogenases. NADP(H), not NAD(H) serves as a cofactor. This enzyme catalyzes the conversion between alcohol and aldehyde. It uses a variety of alcohols, such as ethanol, propanol, butanol, pentanol heptanol, octanol, phenylethanol and aldehydes, such as acetaldehyde and methylgloxal as substrates, indicating it has a wide range of substrate specificity. For oxidation of alcohol, it has an optimal pH of 10.2 with a specific activity of about 56 U/mg protein. However, the reduction of aldehyde has an optimal pH of 7.0. Furthermore, the apparent Km-values for NADPH, NADP, acetaldehyde and alcohol are found to be 0.22 mM, 0.016 mM, 0.3 mM and 2.6 mM, respectively. Therefore, the enzyme likely catalyzes the formation of alcohol in vivo. These results indicate that the alcohol dehydrogenase is a key enzyme in the metabolic pathway of alcohol production in T. hypogea. Further characterization of the enzyme will be carried out.

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A NOVEL FAMILY 8 PSYCHROPHILIC XYLANASE: FUNDAMENTALS AND APPLICATIONS

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Xylanases (EC 3.2.1.8) are O-glycoside hydrolases which cleave the 1,4-â-D-xylosidic linkages of xylan, the major component of plant hemicellulose. Typically xylanases are classified into glycoside hydrolase families 10 and 11 on the basis of amino acid sequence similarities but we have isolated a novel cold-adapted xylanase from an Antarctic bacterium that does not show any homology to members of these families and low homology (20-30% identity) to family 8 members (mainly endoglucanases). This is a very unique enzyme, displaying an $(á/á)_6$ barrel fold and catalysing hydrolysis with inversion of the anomeric configuration, indeed this is the first xylanase shown to display such a structure and mechanism of action and highlights the importance of extremophilic environments as sources of novel enzymes. This enzyme is specific for xylan, is most active on long-chain xylo-oligosaccharides and in contrast to most other xylanases studied to date it is not active on aryl-â-glycosides of xylobiose or xylotriose. It displays typical characteristics for a cold-adapted enzyme: high activity at low temperatures, a reduced thermal and chemical stability and an increased flexibility as compared to a thermophilic homologous enzyme. Structural analysis indicates the basis for this adaptation as being a reduced number of salt bridges and an increased exposure of hydrophobic residues. In addition, crystallographic and site-directed mutagenesis studies have been used to identify the catalytic residues of this enzyme. The utility of this enzyme in the baking industry has also been clearly demonstrated; dough preparation and proofing is generally carried out at temperatures below 35°C and we have shown that 5 to 10 times less of the psychrophilic enzyme is required to give the same desired effect in baking as a commonly used commercial mesophilic xylanase. Indeed, the psychrophilic characteristics (high activity at low to moderate temperatures and low stability) of this enzyme, and of psychrophilic enzymes in general, should be of much benefit in those biotechnological processes carried out at low to moderate temperatures, in particular in the food industry, where its high activity should lead to high yields with low concentrations of enzyme. Here the physicochemical, kinetic, cold adaptation and structural characteristics of this enzyme as well as its applicability for use in industry will be presented.

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IDENTIFICATION AND CHARACTERIZATION OF A NEW TRNA:GUANOSINE-10 DIMETHYLTRANSFERASE IN PYROCOCCUS ABYSSI

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Naturally occurring tRNA in all life forms always contains numerous chemically altered nucleotides. Of the many enzymes that account for these modifications, only a few have been identified so far in Archaea. Here we demonstrate that PAB1283 gene from the hyperthermophilic archaeon P. abyssi encodes a tRNA MTase that comprises a Rossmann-fold catalytic domain in the C-ter and a THUMP, RNA binding domain in the N-ter. The purified recombinant PAB1283 protein behaves as monomeric 39 kDa entity and forms a one to one complex with tRNA. It catalyzes the site-specific, S-AdoMet dependent methylation of exocyclic N2-group of guanosine located at position 10. Depending on the tRNA substrate tested, the enzymatic reaction leads to the formation of either monomethyl- or dimethyl-G10 with transient accumulation of monomethyl-G10. Only tRNAs having a G10•U25 base pair in D-arm together with variable loop of 4 nucleotides allow the formation of dimethyl-G10, while tRNAs harboring a G10•C25 base-pair and/or longer than 4 nucleotides variable loop, allow the formation of monomethyl-G10. This MTase, now renamed Pab-Trm-G10, differs greatly from another earlier characterized tRNA:dimethyltransferase from P. furiosus (Pfu-Trm-G26, alias Pfu-Trm1) both in its amino acid sequence and its catalytic properties

(Constantinesco et al, JMB. 1999, 291: 375-392). At variance with Pab-Trm-G10, Pfu-Trm-G26 catalyzes the formation of dimethyl-G26 only in tRNAs harboring a G10•C25 base pair together with the 5 nucleotides in the variable loop, and only monomethylation of G26 occurs when a G10•U25 base-pair and/or longer than 4 nucleotides variable loop is (are) present in the tRNA. Our results explain why m2G/m22G is present at position 10 or 26, but never at both positions within the same tRNA, at least in the archaeon H. volcanii. Moreover, the co-occurrence of these two MTases in both Archaea and Eukaryota, but not in Bacteria points out a distinct evolution of tRNA maturation strategies between the two former and the later domains of life. The above results obtained for Trm-G10 and Trm-G26 will be compared with those recently obtained for Trm-A58 (alias TrmI) catalysing the formation of N1-methyladenosine at position(s) 58 (and 57) in several tRNAs, an enzyme that is present in most organisms of the 3 biological domains (Droogmans et al, NAR 31:2148-2156, 2003; Roovers et al, NAR 32: 465-476, 2004).

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METABOLITE AND ENZYME PROFILES OF TCA-CYCLE IN *METHANOCOCCOIDES METHYLUTENS*

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Metabolite and enzyme profiles of TCA-cycle in a methylotrophic marine methanogens, *Methanococcoides methylutens* were studied. The pyruvate pool was sufficiently large and higher than that of phosphoenolpyruvate and it is expected as pyruvate is an initial product of the substrate, methanol. The oxaloacetate pool was also higher than that of phosphoenolpyruvate. It indicates that the flux of carbon was towards TCA-cycle. Fumarate pool was about three times higher than that of "-ketoglutarate indicated that "-ketoglutarate was rapidly converted to glutamic acid which was very high in concentration, 6 ì mol g^{-1} wet cells. Surprisingly, supplementation of 2 per cent fumarate in growth medium showed more than two fold higher accumulation of glutamic acid in the cells of *M. methylutens*. Aspartic acid pool was higher as compared to that of oxaloacetate indicating a rapid conversion of oxaloacetate to aspartic acid. These higher contents of glutamic acid and aspartic acid in the soluble pool suggested that glutamic acid and aspartic acid were central to the formation of other amino acids via transaminases. The cell-free extracts of *M. methylutens* contained very high levels of malate dehydrogenase, which catalyzed preferentially the conversion of oxaloacetate to malate. Relatively high levels of malic enzyme, phosphoenol carboxykinase, phosphoenol carboxylase, pyruvate carboxylase, aspartate aminotransferase, alanine dehydrogenase, glutamine-pyruvate aminotransferase, NADPH-glutamate dehydrogenase (reductive amination activity) and glutamate synthase (GOGAT) were also present. Interestingly the activity of "-ketoglutarate dehydrogenase was not detected. Relatively high levels of NADPH-dependent reductive amination activity of glutamate dehydrogenase catalyzing "-ketoglutarate to glutamate were observed as compared to that of other methanogens. Isoenzymes of reductive amination glutamate dehydrogenase, NADH- and NADPH-specific were present and NADPH-glutamate dehydrogenase had three fold higher activity than that of NADH-specific glutamate dehydrogenase. The NADPH-glutamate dehydrogenase was isolated to its apparent homogeneity with a 10 per cent yield and had a molecular weight of about 300 kDa consisted of 6 subunits of 50 kDa. Divalent ions, Mg^{++}, Ca^{++}, Mn^{++}, activated the enzyme whereas ATP, ADP, AMP, succinate, hydroxylamine etc inhibited the activity of this enzyme.

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CHARACTERIZATION OF WILD-TYPE AND MUTANT FORMS OF A COLD-ACTIVE BETA-GALACTOSIDASE FROM AN ANT-ARCTIC ARTHROBACTER ISOLATE PRO-VIDES INSIGHT INTO COLD-ACTIVITY

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We are isolating and characterizing psychrophilic microorganisms and cloning genes encoding cold-active enzymes. In addition to the biochemical characterization of these enzymes, we use genetic engineering approaches to alter and study enzyme properties. One gene, *bga*S, encodes an especially cold-active â-galactosidase and was cloned from the *Arthrobacter* isolate SB, originally isolated from an Antarctic Dry Valley sample. This gene encodes a 1,053 amino acid protein and is most similar to two *lac*Z-like genes, one from the Antarctic *Arthrobacter* sp. C2-2 (71% similar) and the other from *Arthrobacter psychrolactophilus* (66% similar). The purified BgaS enzyme has a very low temperature optimum (more than 35°C below the mesophilic LacZ enzyme from *Escherichia coli*), retains about 50% of its optimal activity at 0°C, requires Mg^{2+} and K^{+} for activity, is inactivated in less than 10 minutes at 37°C, and irreversibly dissociates into monomers above its optimum temperature. These characteristics make the BgaS enzyme an interesting candidate to study the adaptations necessary for low temperature activity. In our attempts to understand these adaptations, we combined directed and random mutagenesis approaches. The directed mutagenesis targeted a residue in the mobile loop of LacZ where amino acid changes altered catalytic activity and thermostability. Similar changes in this region of BgaS reduced activity and one, G803D, eliminated activity. This null mutant was then subjected to random mutagenesis and screened for activity on 5-bromo-4-chloro-3-indolyl-â-D-galactopyranoside (X-Gal). We obtained 28 *E. coli* transformants with some activity restored and the three with the fastest doubling times with lactose as a carbon source were chosen for gene sequencing. Upon analysis of the sequences, we discovered that only two mutations, E229D and V405A, were required to restore activity to the null mutant. A construct containing these three mutations (bgaS6) was made and the resultant enzyme had a catalytic efficiency twice that found for the wild type enzyme at 18°C. The two mutations were then inserted into a wild-type background and the resultant enzyme (BgaS7) was shown to have a catalytic efficiency three times greater than the wild-type enzyme at 18°C. Assays in milk showed that eighty percent of the lactose was hydrolyzed in 3.5, 6.0, and 7.5 hours by BgaS7, BgaS6, and BgaS, respectively. To

help understand what lead to the increased activity, saturation mutagenesis, computer modeling of the structure of BgaS, and microcalrimetry measurements were also performed. The results from these studies suggest that the E229D and V405A mutations cause the formation of a heat labile domain while the original G803D mutation is partially able to stabilize this domain and effect the propensity of its loop to be open or closed.

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AROMATIC RESIDUES LOCATED CLOSELY TO THE ACTIVE CENTER ARE ESSENTIAL FOR THE CATALYTIC REACTION OF FEN-1 FROM HYPERTHERMOPHILIC ARCHAEON PYROCOCCUS HORIKOSHII

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Flap endonuclease-1 (FEN-1) is a structure-specific nuclease that is central to both DNA replication and repair processes. The FEN-1 class of structure-specific 5' nuclease occurs in all domains of life. The crystal structure of FEN-1 from Pyrococcus horikoshii was determined to a resolution of 3.1 Å. In this study, the kinetic parameters of mutants at highly conserved aromatic residues, Tyr33, Phe35, Phe79, and Phe278Phe279, in the vicinity of the catalytic centers of FEN-1 were examined. The substitution of these aromatic residues with alanine led to a large reduction in kcat values, although these mutants retained Km values similar to that of the wild-type enzyme. Notably, the kcat of Y33A and F79A decreased 333-fold and 71-fold, respectively, compared with that of the wild-type enzyme. The aromatic residues Tyr33 and Phe79, and the aromatic cluster Phe278Phe279 mainly contributed to the recognition of the substrates without the 3' projection of the upstream strand (the nick, 5'recess-end, single-flap, and pseudo-Y substrates) for the both exo- and endo-activities, but played minor roles in recognizing the substrates with the 3' projection (the double flap substrate and the nick substrate with the 3' projection). The replacement of Tyr33, Phe79 and Phe278Phe279, with non-charged aromatic residues, but not with aliphatic hydrophobic residues, recovered the kcat values almost fully for the substrates without the 3' projection of the upstream strand, suggesting that the aromatic groups of Tyr33, Phe79, and Phe278Phe279 might be involved in the catalytic reaction, probably via multiple stacking interactions with nucleotide bases. The stacking interactions of Tyr33 and Phe79 might play important roles in fixing the template strand and the downstream strand, respectively, in close proximity to the active center to achieve the productive transient state leading to the hydrolysis.

1. E. Matsui, S. Kawasaki, H. Ishida, K. Ishikawa, Y. Kosugi, H. Kikuchi, Y. Kawarabayashi, and I. Matsui. "Thermostable Flap Endonuclease from the Archaeon, Pyrococcus horikoshii, Cleaves the Replication Fork-like Structure Endo/Exo nucleolitically" (1999) J. Biol. Chem., 274, 18297-18309.
2. E. Matsui, K. V. Musti, J. Abe, K. Yamasaki, I. Matsui, and K. Harata "Molecular structure and novel DNA binding sites located in loops of flap endonuclease-1 from Pyrococcus horikoshii" (2002) J. Biol. Chem., 277, 37840-37847.
3. E. Matsui, J. Abe, H. Yokoyama, and I. Matsui "Aromatic Residues Located Closely to the Active Center are Essential for the Catalytic Reaction of FEN-1 from Hyperthermophilic Archaeon Pyrococcus horikoshii " (2004) J. Biol. Chem., in press.

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TOPOTAQ DNA POLYMERASES FOR GENE TECHNOLOGIES: PROCESSIVE, HYPERSTABLE, AND RESISTANT TO COMMON INHIBITORS

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We have developed a new technology for production chimeric DNA polymerases with outstanding thermostability, processivity, strand displacement ability, and resistance to common inhibitors of DNA polymerases. The chimeras contain polymerase domains fused with helix-hairpin-helix (HhH) domains derived from topoisomerase V of *Methanopyrus kandleri*. The advantages of new polymerases allowed for cycle sequencing and PCR in high salts and at temperatures inaccessible for other DNA polymerases. Using this technology, we engineered a TOPOTAQ series of DNA polymerases, which are extremely resistant to common inhibitors of DNA polymerases and present an excellent choice for DNA amplification in samples with intercalating dyes (SYBR green, SYBR gold, ethidium bromide, indigo), organic solvents (phenol), and physiological fluids (such as blood, urine, and others). The enzymes are very efficient for sequencing and PCR amplification from bacterial cultures, colonies and other crude samples, for high-fidelity amplification, and for amplification of GC-reach DNAs. The chimeric polymerases can also be used together with the commercial BDT sequencing kits to sequence modified bacteriophage DNAs or in case of structured DNA templates that produce stops in DNA sequence readings.

Supported in part by DOE and NIH.

1. Pavlov *et al.*, (2004) *Thermostable chimeric DNA polymerases with high resistance to inhibitors*. In: DNA Amplification: Current Technologies and Applications (eds. Demidov, V. & Broude, N.) Horizon Bioscience, pp 3-20.
2. Pavlov *et al.*, (2004) *Recent developments in the optimization of thermostable DNA polymerases for efficient applications*. Trends in Biotechnology. **22**: 253-260.
3. Pavlov *et al.*, (2002) *Helix-Hairpin-Helix motifs confer salt resistance and processivity on chimeric DNA polymerases*. Proc. Natl. Acad. Sci. USA. **99**: 13510-5.

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HIGH THROUGHPUT COMPLETE DIRECT SEQUENCING OF PHAGE AND MICROBIAL GENOMES

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We have developed tools and workflow for direct genomic DNA sequencing that eliminates the need in shotgun libraries, minimizes the number of sequencing reactions and dramatically accelerates finishing of complete genomes. Other advantages of direct sequencing are the elimination of artifacts of library or PCR cross contamination in simultaneous production sequencing of related organisms and significant savings on data processing due to non-biased complete and low coverage of the genome. ThermoFidelase and D-Strap Fimers contribute to cost savings and speed up sequencing of closely related genomes. Using the directed sequencing strategy, we have finished 15 prokaryotic genomes (genome size 1.7-4.5 Mb) and over 10 phage genomes (average genome size 170 kb) to the publication quality during the last two years. The individual elements of the finishing strategy will be illustared using these data.

Supported in part by DOE and NIH.

1. Malykh *et al.*, (2004) *Finishing "Working Draft" BAC Projects by Directed Sequencing with ThermoFidelase and Fimers*. In: Methods in Molecular Biology, (eds. Stodolsky, M. & Zhao, S.). Vol. Bacterial Artificial Chromosomes: Methods and Protocols, pp. 295-308.
2. Malykh *et al.*, (2004) *Use of Fimers to Eliminate Polymerase Chain Reaction and Primer-Dimer Artifacts and to Increase Yield in BAC sequencing reactions*. In: Methods in Molecular Biology, (eds. Stodolsky, M. & Zhao, S.). Vol. Bacterial Artificial Chromosomes: Methods and Protocols, pp. 221-30.
3. Polushin *et al.*, (2004) *High-Throughput Production of Optimized Primers (Fimers) for Whole-Genome Direct Sequencing* In: Methods in Molecular Biology, vol. 288: Oligonucleotide Synthesis: Methods and Applications. pp. 291-304, Herdewijn P (Ed). Humana Press.
4. Slesarev *et al.*, *The Complete Genome of the Hyperthermophile Methanopyrus kandleri AV19 and Monophyly of Archaeal Methanogens*. (2002) Proc. Natl. Acad. Sci. USA. **99**: 4644-9.

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MICROBIAL POPULATION OF TEXTILE EFFLUENTS - A COUNTERACTIVE TOOL FOR POLLUTION CONTROL

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Textile effluents are a rich source of diversified microbes adapted to survive under extreme condition in terms of unusual nutrients available, pH and temperature. A textile effluent is a potential source for the isolation of bacterial population capable of degradation of almost all textile dyes due to its adaptation to that environment. In the present study we tested 55 bacterial isolates obtained from textile effluents for the decolorization and degradation of various textile dyes under normal to extreme physico chemical like temperature, pH etc. Result obtained indicates that these some of these isolates can be useful to exploit for bioremediation to clean up polluted environment, which is otherwise harmful to the human health and agriculture.

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DISTRIBUTION OF THE NOVEL PHYLUM NANOARCHEOTA IN THERMAL BIOTOPES

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We conducted an extensive survey of thermal biotopes using the nanoarchaeal-specific 16S 7mcF and 1511mcR primers to detect novel nanoarchaeal 16S rRNA genes. We identified 14 novel nanoarchaeal 16S sequences in thermal samples from Yellowstone National Park sediments, mud from Indonesia, soil from Kamchatka peninsula, Russia, and sediments from Iceland. All these samples had temperatures ranging from 70°C to 97°C. The nanoarchaeal 16S sequences identified form a cluster with the sequence of Nanoarchaeum equitans, but no new sequence was identical to it, indicating that that there is a great deal of genetic diversity within the Nanoarchaeota lineage. Most of the sequences were clustered by locations and the Yellowstone sequences found in this study clustered with the only nanoarchaeal sequence from Yellowstone OP9 deposited in Genebank. These results will help us to design new primer sets for Nanoarchaeota species, and to understand their distribution in other thermal and non-thermal biotopes.

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NOVEL THERMOPHILIC ENZYMES AND THEIR APPLICATION IN BIOCATALYSIS

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Hyperthermophiles are an ideal source of novel stable enzymes that have applications as biocatalysts for the synthesis of optically pure compounds of importance to the pharmaceutical industry. The Exeter group has isolated and studied several novel enzymes from the hyperthermophilic archaea, *Thermococcus litoralis*, *Pyrococcus furiosus*, *Solfolobus solfataricus* and *Aeropyrum pernix*. The enzymes have been cloned and over-expressed in *Escherichia coli* and characterised regarding their biochemical properties, 3D structure and substrate specificities. The enzymes to be discussed will be the *T.litoralis* pyroglutamyl carboxyl peptidase (Pcp) and L-aminoacylase, the *P.furiosus* lysophosphlipase, the *S. solfataricus* gamma lactamase/amidase and the *A. pernix* alcohol dehydrogenase (ADH). The *T.litoralis* Pcp is a novel cysteine peptidase classified as an omega (ù) peptidase (EC 3.4.19.3). The enzyme hydrolytically removes the N-terminal pyroglutamate (pGlu) residue of pGlu peptides and proteins with selectivity towards *L*-pGlu-*L*-amino acid optical isomers. We have solved the structure of this enzyme and its specificity has been studied. The *pcp* gene is located upstream of the gene encoding a novel L-aminoacylase. The *P.furiosus*

lysophosphlipase belongs to the hydrolase family of enzymes that act upon carboxylic esters and has been shown to catalyse the reaction between 2-phosphatidylcholine and water to generate glycerolphosphcholine and a fatty acid anion. This enzyme has sequence similarity to other LysoPL's from hyperthermophilic archaea and shares 33% identity to a human monoglyceride lipase. There is no known structure for this class of lipase enzyme and crystallization studies are in progress. The *S. solfataricus* gamma lactamase/amidase is related to the signature amidase family and can be used for the production of carbocyclic sugars that can be built into carbocyclic nucleosides that are potent anti-viral compounds. The *A. pernix* ADH has interest industrially for the production of optically pure alcohols. The structure of the enzyme in the holo form with a bound inhibitor octanoic acid has been solved. This ADH shares 24-25% identity with the eukaryotic horse liver and human ADH enzymes. The identity to the thermophilic eubacterium *Thermoanaerobium brockii* ADH is 28% and is lower when compared with other bacterial species. The *Aeropyrum* ADH is very thermostable has a half-life of over 2hours at 90°C.

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GENESTYLES OF THE HOT AND SINGLE: GENOMIC ANALYSIS OF INDIVIDUAL CELLS FROM OBSIDIAN HOT SPRING

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Constructing genomic libraries from extreme environmental niches is challenging due to the low abundance of microbes; for example, as few as 1,000 cells per ml are found in some thermal aquifers, yielding only picogram amounts of DNA. Metagenomic library construction methods result in fragmented sequence information that is limited to the contents of the insert. Assembling large contiguous portions of a genome will yield important insights into the physiology, ecology, and metabolic strategies used by uncultured microorganisms. "Single Cell Genomics" is a new initiative to develop the tools and methods to sequence the genome of individual cultivation-resistant microbes. This technology facilitates comprehensive expression screening and complete analysis of the genetic diversity of microbial communities. Single microorganisms are manually isolated from a diverse mixture using a micromanipulator. The genomic DNA is then randomly amplified and cloned into a high-stability, transcription-free vector. Using this method we generated 10X genomic coverage of the model organism *Pyrococcus furiosus*, whose complete genome sequence is available. Sequence analysis of 384 clones from this library showed that the clones were randomly distributed throughout the genome, with no apparent clustering, bias, or lack of fidelity. We are using these methods to study the microorganisms from Obsidian and Bath hot springs in Yellowstone National Park. Obsidian hot springs has been previously shown to contain 12 novel, division level lineages; Bath is a boiling thermal pool that has not been studied at the genomic level. We will present preliminary genomic data from microorganisms isolated from these environments.

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TEMPERATURE AND NUTRIENT AVAILABILITY CONTROL GROWTH RATE AND FATTY ACID COMPOSITION OF FACULTATIVELY PSYCHROPHILIC COBETIA MARINA STRAIN L-2

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A facultative psychrophilic bacterium, strain L-2, that grows at 0°C and 5°C as minimum growth temperatures in complex and defined media, respectively, was isolated. On the basis of taxonomic studies, strain L-2 was identified as Cobetia marina. The adaptability of strain L-2 to cold temperature was higher than that of the other reported strains of the same species. When the bacterium was grown at 5-15°C in a defined medium, it produced a high amount oftrans-unsaturated fatty acids, while it produced a low amount of trans-unsaturated fatty acids in a complex medium in the same temperature range. In the complex medium at 5°C, the bacterium exhibited a threefold higher growth rate than that obtained in the defined medium. A notable difference in proportion in growth rate reduction from 11°C to 5°C was observed between the complex and defined media, and an upshift of growth rate considering the proportional decrease in this temperature range was observed in the complex medium. Furthermore, pronounced enhancements in growth rate and yield were observed in growth temperature between 0°C and 5°C when strain L-2 was grown at complex medium. These phenomena might indicate certain enhancement of metabolism occurred at 5°C. The effects of adding complex medium components to the defined medium on bacterial growth rate and fatty acid composition at 5°C were observed. The addition of yeast extract followed by peptone was effective in promoting rapid growth, while glutamate addition was less effective. Glutamate addition to the defined medium resulted in a cis-unsaturated fatty acid ratio similar to that of the cells grown in the complex medium. These results suggest that for the rapid growth of strain L-2 at low temperatures, a high content of various amino acids rather than a high ratio of cis-unsaturated fatty acids in their membrane is required.

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METABOLIC AND PHYLOGENETIC DIVERSITY OF THERMOPHILIC HYDROGENOGENIC CO-OXIDIZING PROKARYOTES

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Distribution, phylogeny and metabolic diversity of hydrogenogenic CO oxidizing anaerobes were studied. Two new bacterial genera *Thermoincola* gen. nov. and *Thermosinus* gen. nov. were isolated from terrestrial hot springs. Both organisms were shown to be moderate

thermophiles and obligate anaerobes oxidizing CO and producing equimolar quantities of H_2 and CO_2. *Thermoincola carboxydophila* sp. nov. was shown to be obligate chemolithotroph and, as the previously known microorganisms of this group, did not reduce any electron acceptors. *Thermosinus carboxydivorans* sp. nov. was shown to be facultative carboxydotroph capable of organotrophic growth. During the growth on CO it was able to reduce Fe(III) to Fe(II) and selenite to elemental selenium, but could grow without electron acceptors. Contrary to it, a new hydrogenogenic carboxydotrophic isolate obtained from Kamchatka turned to be obligately dependent on the reduction of ferric iron. From hydrothermal venting structures collected at East Pacific Rise 13°N a hyperthermophilic archaeon capable of lithotrophic growth on CO coupled with equimolar production of molecular hydrogen was isolated. The growth of the new isolate on CO was stimulated by thiosulfate which was reduced to H_2S. Based on its 16S rRNA sequence analysis, this organism was affiliated to the genus *Thermococcus*. This is the first evidence of anaerobic CO oxidation coupled with H_2 production performed by hyperthermophilic Archaea.

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SCREENING OF ACIDOTHERMOPHILIC AUTOTROPHES FOR METAL ADSORPTION AND FOR CHALCOPYRITE,COVELLITE AND PYRROHOITITE BIOLEACHING POTENTIALITY FROM WESTERN INDIA

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Microbial processes are gaining increasing interest in the mining industry. In bacterial leaching, the solubilization is mediated by bacteria. The main advantages of bioleaching as compared with pyrometallurgy lie in its relative simplicity, mild operation conditions, low capital costs, low energy input, and in its friendliness towards the environment. A wider temperature range can be very important for organisms in various ways, since it makes an organism more versatile with regard to changes in the environment. Acidothermophilic chemoautotrophic organisms are potentially well suited to industrial leaching applications where considerable temperature fluctuations limit the growth of other non-thermophilic bioleaching microorganisms. Having developed an interest in these unusual microbes, I started to work in this area. In Western India, Rajkot City in the state of Gujarat is metallurgically very active since decades. Many industries like metal finishing industries, gold and silver refining industries, petroleum refining, iron and steel industries use copper, zinc, chromium, silver, lead and other heavy metals as important ingredients to develop various types of metal alloys. The screening was carried out for chalcopyrite, covellite and pyrrohoitite biosolubilizing potency as well as for metal tolerance in modified 9K medium at 2.5 pH, 60^0 C temperature on environmental shaker. The experiments were periodically repeated by adding multi heavy metals at higher gradient of concentration of Ag, As, Bi, Cd, Cr, Co, Cu, Hg, Li, Mo, Pb, Sn and Zn from 10^{-7} to 10^{-3} M to induce multi metal resistance as well as metal absorption efficiency. The selected highly potential isolates were analyzed with Energy Diffraction Assay X-ray Crystallography EDAX for metal biosorption data. Isolate ***Th-KV$_2$***, one of the best, giving 68% solubilization value as ferrous sulfate and 40% value was observed to be getting adsorbed as soluble copper from chalcopyrite ore on the fifth day of the experiment. Further studies are in progress; the comparative ore leaching and biosorption ability of these unidentified isolates have been described.

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MICROBIAL ACTIVITY BELOW FREEZING POINT

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Recent progress in research on life in extreme environments made it clear that some microorganisms are able to metabolize at extremely low temperatures. Frozen habitats accommodating such microorganisms include refrigerated food, permafrost and seasonally frozen polar soils, mountainous glaciers, Arctic/Antarctic ice and, probably, extraterrestrial objects within the Solar System and outside. The most intensive studies were performed last decade with natural polar ecosystems (Arctic and Antarctic) dominated by permafrost and oceanic ice. It was found the sizeable winter CO2emission from tundra to atmosphere caused by slow release of summer-accumulated gases and by the cold-season respiratory activity of the indigenous soil/permafrost microorganisms [1, 2]. The present communication summarizes our kinetic and molecular data on the nature of CO2 generation in permafrost. Frozen tundra (Barrow) samples were incubated at 0 - -40°C with added 13/14C-subatrates, and microbial activity was recorded as: a) the rates of CO2 and 14CO2 evolution; b) dark 14/13CO2 assimilation (anaplerotic reactions related to growth); c) the incorporation of 13/14C-precursors into cellular DNA, proteins and phospholipids; d) separation and sequencing of heavy 13C-DNA to identify responsible organisms (in progress). We have found that 'soil respiration' alone can be misleading indicator of microbial activity due to slow continuous release of CO2 tightly bound to solids. The corrected microbial respiration (net flux - flux from poisoned sample) was detectable at all tested temperatures down to -40 (!) and displayed double exponential dependence on temperature and unfrozen water content. Kinetic analysis of dynamic data [3] allowed us to assess population density and major physiological characteristics of endogenous psychrophiles (growth rate, yield, turnover, maintenance, hydrothermal parameters, etc) and optimize their isolation procedure. The ability of arctic psychrophiles to metabolize below zero reinforces the possibility to find life outside the Earth.

1Panikov, N.S. & Dedysh, S.N. 2000. GBC **14,** 1071.
2Panikov, N.S. & Mastepanov, M.M. 2001. 3d Int. Conf. Cryopedology 2001.
3Panikov N.S. 1995. Microbial Growth Kinetics. Chapman & Hall, L.

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REHYDRATION OF THE NATURALLY DESICCATED LICHEN *RAMALINA LACERA* RESULTS IN PRODUCTION OF ROS AND RNS AND ALTERATIONS IN ANTIOXIDANTS

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Lichens are symbiotic associations of fungi and unicellular green algae or cyanobacteria. The lifestyle of most lichens is composed of alternating periods of desiccation with low metabolic activity, and hydration that induces increase in their metabolism. The effect of rehydration on ROS (reactive oxygen species) and RNS (reactive nitrogen species) production and ROS-related parameters was investigated in the lichen *Ramalina lacera*. Rehydration of *R. lacera* resulted in initiation of- and rapid increase in photosynthesis, which was accompanied by bursts of ROS and NO production as assessed by dichlorofluorescein (DCF) or diaminofluorescein (DAF-2), respectively. The rate and extent of ROS production was similar in the light and in the dark, suggesting a minor contribution of photosynthetic processes to the total production of these oxygen metabolites. Laser-scanning confocal microscopy of DCF revealed that ROS formation following rehydration was associated with both symbiotic partners, while DAF-2 fluorescence, indicating NO formation, was detected only in fungal hyphae. The burst of ROS production was accompanied by membrane damage seen as an increase in ion-leakage from the thalli. Native gel electrophoresis of crude extracts of *R. lacera* revealed ten bands of SOD activity, and two bands of catalase activity. Eight of the SOD bands that were identified as 4 Fe-SOD and 4 Mn-SOD are synthesized by the alga, and two bands, a Cu/Zn-SOD and a Mn-SOD are the product of the fungus. The major catalase band is produced by the fungus and the algal catalase activity is much lower. After rehydration there was a decrease in the activities of all SODs, catalase, glutathione reductase and glucose 6-phosphate dehydrogenase. Total water-soluble, low-molecular weight antioxidants decreased following rehydration, while total lipid-soluble antioxidants increased.

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HIGH TEMPERATURE COMPOST: A NEW HABITAT OF EXTREME THERMOPHILES

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Most of extreme thermophiles, both bacterial and archaeal, have been isolated from hydrothermal environments such as hot springs and thermal vents on the deep sea floor. Compost was most important source of isolating many moderate thermophiles in the classical era of the study of thermophiles. Recently "high temperature" compost has been developed, of which inside temperature reaches up to 95°C or even higher (inside of the conventional compost is below than 80°C). We have isolated many extreme thermophiles from one of the high temperature compost in Japan. One of the major species, strain YMO81, is an aerobic, long rod, and is capable of growing at 83°C. Though 16S rDNA base sequence of YMO81 showed similarity (about 90%) to those of *Bacillus stearothermophilus*, the isolate could be classified into a new genus because YMO81 is non-spore forming, and gram negative. Electron microscopic observation confirmed the presence of an outer membrane. Phylogenetic analysis showed the isolate is independent from Bacillus-Clostridium species suggesting the YMO81 belongs to a new genus. YMO81 could grow in a synthetic medium at relatively lower temperatures, but required the addition of essential amino acids for growth at high temperature extreme. Some strains produce heat stable protease(s). We also isolated many lipase-producing thermophiles from high temperature compost. Purified lipases were highly resistant to heat as expected. The high temperature compost would be a promising source for isolating new extreme thermophiles.

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BLACK YEAST HORTAEA WERNECKII - A NEW EUKARYOTIC HALOPHILIC MODEL ORGANISM

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Hypersaline environments are inhabited by bacteria, few algal species and a new group of eukaryotic extremophiles represented by melanized fungi, so called black yests. The dominant species among them is the extremely halophilic Hortaea werneckii, with the capability of growing in media with no salt and up to 5.2 M NaCl concentration. It represents an appropriate model organism to study molecular mechanisms of salt tolerance in eukaryotes. Cellular responses to high salinity were investigated in H.werneckii at the level of expression of salt responsive genes and fuctions of their their products. By using a restriction fragment-differential display method we identified eight differentially expressed genes in response to different salt concentration. Functions of their putative products within cellular machinery was proposed. An increase in intracellular glycerol concentration was observed at increased environmental salinities in H. werneckii. Expression profile of a gene, coding for a putative glycerol-3-phosphate dehydrogenase (Hwgpd), a key enzyme in glycerol biosynthesis, revealed several-fold increased expression at high salt concentration. This increased expression of Hwgpd gene correlated with the increased activity of HwHog1, a key kinase of HOG signalling pathway, which senses and responds to hyperosmotic stress, and regulates the expression of gpd genes. Since glycerol is abundant at higher salinities, we speculated, that relatively less metabolic flux remains for the late stages of glycolysis and for the Krebs cycle. This assumption was supported with our finding, that production of CO2 was diminished at higher salt concentration. Increased expression of SOL5, a putative aconitase gene at increased salinity could also be related to this phenomenon. No major changes in intracellular ion concentration occur in H. werneckii with the increased external salinity. Although Na+ to K+ ratio increases with increased NaCl concentration in the medium, it does not follow the external Na+ concentration. These data suggest, that halophilic H. werneckii is well

adapted to changes in external ion concentrations, most probably due to its ability to effectively extrude Na+ ions. Expression profile of two genes, that could be connected to Na+ transport, SOL4 and homologue of ENA1, support this hypothesis. Co-ordinated expression of salt-stress responsive genes, correlated to their putative functions, have so far revealed some mechanisms that enable H. werneckii to thrive at extremely high NaCl concentration and to adapt to a wide range of NaCl concentrations in its environment.

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ACCUMULATION OF CADMIUM IONS (CD+2) BY HALOARCHAEON GUSF, AN ISOLATE FROM SALT PANS OF GOA - INDIA

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GUSF (MTCC 3265) a haloarchaeon isolated from salt pans of Goa - India, and characterized as *Haloferax spps* was studied for its tolerance to cadmium (Cd^{+2}) ions. The haloarchaeon tolerates a maximum of 4 mM Cd^{+2} during its growth in tryptone yeast extract medium with 25% NaCl and and 2 mM Cd^{+2} during its growth in mineral salts glucose medium containing 25% NaCl. Growth in both media is unaffected in presence of a maximum of 1 mM Cd^{+2}. Growing cells show accumulation of Cd^{+2}, detectable by X-ray analysis and complexation with dithizone. A 60% of accumulated Cd^{+2} is associated with the cell envelope and remaining could be recovered from cytosol fractions. Accumulation of Cd^{+2} by GUSF cells is associated with induction of a low molecular weight glycoprotein.

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STABILITY AND FOLDING OF DIII FROM E.COLI TRANSHYDROGENASE IN THE PRESENCE OF NADPH OR NADP

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A rapidly growing research field of today concerns protein stability and how a protein folds into a stable and functional structure. In the present investigation the stability and folding properties of the soluble domain dIII of nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) from E. coli were determined. The processes were investigated in the presence of two different substrates, NADPH and NADP. Nicotinamide nucleotide transhydrogenase (TH) is a membrane-bound enzyme located in the cytoplasmic membrane of bacteria and in the inner membrane of the mitchondria. The enzyme catalyses the reversible reduction of NADP+ by NADH coupled to a proton translocation across the membrane. TH consists of three domains, domain I and III that binds NAD(H) and NADP(H), respectively, and the proton translocating, membrane spanning domain II. The NADP(H) binding domain of E. coli TH, ecIII, contains 177 amino acids essentially adopting an nucleotide-binding fold. The structure is composed of an open twisted six-stranded parallel beta-sheet which is flanked by helices on both sides of the sheet. The stability and folding properties of ecIII+NADPH or NADP have not been studied previously and, furthermore, the protein also represents a generally interesting case for investigating the correlation between ligand binding affinity and protein stability. We demonstrate that the higher affinity for NADPH is reflected as a slightly higher stability for the ecIII+NADPH complex compared to the ecIII+NADP complex, as indicated by the free energy of folding and midpoint of transition. However, the exposed surface area upon transition is not changed whether NADPH or NADP is bound. We also conclude that the parameters describing the stability and folding/refolding properties of ecIII are independent of the methods of use, i.e. far UV- circular dichroism (CD) or intrinsic tryptophan fluorescence. Furthermore, characterisation by differential scanning calorimetry has demonstrated that the unfolding of ecIII is reversible and ecIII+NADPH has a slight increased thermal stability compared to ecIII+NADP.

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AROMATIC RESIDUES LOCATED CLOSELY TO THE ACTIVE CENTER ARE ESSENTIAL FOR THE CATALYTIC REACTION OF FEN-1 FROM HYPERTHERMOPHILIC ARCHAEON PYROCOCCUS HORIKOSHII

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Flap endonuclease-1 (FEN-1) is a structure-specific nuclease that is central to both DNA replication and repair processes. The FEN-1 class of structure-specific 5' nuclease occurs in all domains of life. The crystal structure of FEN-1 from Pyrococcus horikoshii was determined to a resolution of 3.1 Å. In this study, the kinetic parameters of mutants at highly conserved aromatic residues, Tyr33, Phe35, Phe79, and Phe278Phe279, in the vicinity of the catalytic centers of FEN-1 were examined. The substitution of these aromatic residues with alanine led to a large reduction in kcat values, although these mutants retained Km values similar to that of the wild-type enzyme. Notably, the kcat of Y33A and F79A decreased 333-fold and 71-fold, respectively, compared with that of the wild-type enzyme. The aromatic residues Tyr33 and Phe79, and the aromatic cluster Phe278Phe279 mainly contributed to the recognition of the substrates without the 3' projection of the upstream strand (the nick, 5'recess-end, single-flap, and pseudo-Y substrates) for the both exo- and endo-activities, but played minor roles in recognizing the substrates with the 3' projection (the double flap substrate and the nick substrate with the 3' projection). The replacement of Tyr33, Phe79 and Phe278Phe279, with non-charged aromatic residues, but not with aliphatic hydrophobic residues, recovered the kcat values almost fully for the substrates without the 3' projection of the upstream strand, suggesting that the aromatic groups of Tyr33, Phe79, and Phe278Phe279 might be involved in the catalytic reaction, probably via multiple stacking interactions

with nucleotide bases. The stacking interactions of Tyr33 and Phe79 might play important roles in fixing the template strand and the downstream strand, respectively, in close proximity to the active center to achieve the productive transient state leading to the hydrolysis.

1. E. Matsui, S. Kawasaki, H. Ishida, K. Ishikawa, Y. Kosugi, H. Kikuchi, Y. Kawarabayashi, and I. Matsui. "Thermostable Flap Endonuclease from the Archaeon, Pyrococcus horikoshii, Cleaves the Replication Fork-like Structure Endo/Exo nucleolitically" (1999) J. Biol. Chem., 274, 18297-18309.
2. E. Matsui, K. V. Musti, J. Abe, K. Yamasaki, I. Matsui, and K. Harata "Molecular structure and novel DNA binding sites located in loops of flap endonuclease-1 from Pyrococcus horikoshii" (2002) J. Biol. Chem., 277, 37840-37847.
3. E. Matsui, J. Abe, H. Yokoyama, and I. Matsui "Aromatic Residues Located Closely to the Active Center are Essential for the Catalytic Reaction of FEN-1 from Hyperthermophilic Archaeon Pyrococcus horikoshii " (2004) J. Biol. Chem., in press.

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ACQUISITION OF DEEP-SEA ORGANISMS AND PRESSURE ENVIRONMENT CONTROL FOR THE INCUBATION TANK

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The adaptation characteristics of extremophiles are quite clear in molecular biology. Especially, the response of piezophiles to a pressure, expression of genes, inhibition of channel proteins, the abnormalities in cell form, etc. are observed. The response in the extremity environment of a multicellular organism have not been explained. We showed that the tissue culture cell of a vertebrate caused a deformation with a pressure. However, there is still no research of change by the pressurization using the multicellular organism which inhabits deep-sea environment. It is a reason that the sample capture method, breeding onboard, and handling method of a deep-sea multicellular organism are not enough examined. Survival sampling is not still easier. Those reasons are the serious hindrance to making progress to tissue culture research. We think that the living cultured cell in which dynamic measurement is possible is required, in order to clarify specificity of a deep-sea multicellular organism. It is required to establishment of a cultured cell-line. Then, we developed the equipment (DEEPAQUARIUM) which can sample a deep-sea multicellular organism, with on-site environment retained. Furthermore, the transfer method in the state where the deep-sea multicellular organism was living was considered. We aimed at trying the tissue culture of a cell. This report indicates that the deep-sea samples which die by the temperature rise or pressure reduction have improved the probability of survival by maintenance of the equipment environment. We report having tried primary culture from the tissue further. This capture machine preserves the deep-sea environment to the depth of 6000m, and aims at capture with a survival. The captured sample was conveyed to the chamber by the connected suction pump, and the manipulator closed the valve of the chamber. The collected capture chamber was connected to the circulating water tank system with the high pressure maintained. As a result of short-term breeding of a deep-sea eel on the ship, when oxygen supply and wastes removal were fully performed, it turned out that a sample can be retained. We will systematize sampling method and try to improve the probability of survival of the deep-sea samples to capture.

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RECOMBINATION/REPAIR IN ARCHAEA : THE RAD50-MRE11-NURA-HERA PATHWAY

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The Rad50-Mre11 pathway may play a crucial role in Recombination/Repair in Archaea. In particular, as in the case of Eucarya, no homologs to the two major bacterial complexes RecBCD and RecFOR involved in the resection of DNA double-stranded breaks at the initiation step of homologous recombination are found in Archaea, and the Rad50-Mre11 complex could be involved in such a process. In all organisms studied so far, the Rad50-Mre11 complex exhibits single-stranded endonuclease and 3' to 5' exonuclease activities as well as a predicted mechanical function inherent to the Rad50 protein. These activities do not allow to understand how the complex can process DNA breaks in 3' single-stranded DNA tails which are necessary for the loading and the activity of the recombinase suggesting that other partners might participate to this step. We showed that in most thermophilic archaea, the rad50-mre11 genes are organized in an operon structure with two other genes of unknown function and we characterized each gene product from Sulfolobus acidocaldarius species. The first gene encodes a single-stranded endonuclease as well as a 5' to 3' exonuclease and defines a new family of nucleases that we called nurA for "nuclease repair of Archaea" (Constantinesco et al, 2002). The second gene called herA for "helicase repair of Archaea" encodes a new type of DNA helicase which possesses the striking property to utilize both 3' and 5' single-stranded DNA extensions for loading and subsequent DNA duplex unwinding (Constantinesco et al, 2004). These results indicate that the archaeal Rad50-Mre11 complex acts in concert with a 5' to 3' exonuclease (NurA) and a bipolar DNA helicase (HerA) confirming a probable involvement in the initiation step of homologous recombination. The characterization of the concerted action of the Rad50-Mre11-NurA-HerA proteins is presently in progress in our laboratory.

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ISOLATION AND CULTURE OF CARBON MONOXIDE DEPENDENT THERMOPHILES AND HIGH TEMPERATURE PHOTOSYNTHETIC MATS FROM THE UZON CALDERA, KAMCHATKA

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The geothermal sites of the Uzon Caldera, Kamchatka, provide unique opportunities to determine the relationship between microbial and geochemical transformations during mineral formation. In this study, two sites within the Uzon Caldera have been characterized, using microbial culturing and detection techniques. A bacterial mat from the K4 well area in the Central Thermal Field of the caldera was transported to US laboratories, and successful cultures of the mat were maintained at 45, 50 and 55^{0}C. The bacterial populations of the mats were analyzed using denaturing gradient gel electrophoresis (DGGE), culture and isolation of bacterial strains, and cloning and sequencing of 16SrRNA from isolates and the mat populations. In addition, hot spring sediment and the mat cultures were subcultured into anaerobic media that contained CO as the sole carbon and energy source. Isolates obtained were anaerobic, CO-oxidizing thermophiles that clearly survived transportation of the mat at room temperature from Russia under aerobic conditions, and were then established in consortial growth of the mats. DGGE revealed that the isolates were represented in the mat bacterial population, and DNA samples extracted from original mat material in the field. The cultured mat was less diverse than the original mat sample, however many shared bands were observed. Sequencing of full length 16S rDNA clones revealed that the mat population contained several novel bacterial species, and was dominated by Chlorogloeopsis sp. (51% of the sequenced clones). The presence of novel carboxydotrophic bacteria will allow us to address the functions of strictly anaerobic, hydrogenogenic bacteria in these mats that are energized by oxygenic photosynthesis.

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COMPARISON OF 16S RRNA GENE SEQUENCES OF THE GENUS *METHANOBREVIBACTER*

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The phylogeny of the genus *Methanobrevibacter* was established almost 25 years ago on the basis of the similarities of the 16S rRNA oligonucleotide catalogs. Since then, many 16S rRNA gene sequences of newly isolated strains or clones representing the genus *Methanobrevibacter* have been deposited in the public databases. In addition to 16S rRNA gene sequence similarity (*S*), genomic DNA reassociation (*D*) values are also essential for the correct identification of a strain. The statistical implications of the correlation between these two parameters are of great interest in prokaryotic systematics and it depends on the taxa in study. We therefore revised the taxonomic affiliation of the isolates and clones of the genus *Methanobrevibacter,* based on 16S rRNA gene sequences, deduced group specific nucleotide positions showing specific nucleotide substitutions in the 16S rRNA gene sequences, and studied the correlation between *D* and *S* based on the available DNA hybridization data. Our analysis based on 786 bp aligned region from 54 representative sequences of the 120 sequences available for the genus revealed seven multi-member groups namely, Ruminantium, Smithii, Woesei, Curvatus, Arboriphilicus, Filiformis, and the Termite gut symbionts along with three separate lineages represented by *Mbr. wolinii, Mbr. acididurans,* and termite gut flagellate symbiont LHD12. The cophenetic correlation coefficient (0.913), a test for the ultrametric properties of the 16S rRNA gene sequences used for the analysis, indicated the high degree of goodness of fit of the tree topology. A significant relationship was found between *S* and *D* with the correlation coefficient (*r*) for log*D* and log*S*, and for [ln(-ln*D*) and ln(-ln*S*)] being 0.73 and 0.796 respectively. Our analysis suggested that D would be less than 70 % at least 99 % of the times when *S*= 0.984, and with 70% *D* as the species "cutoff", any 16S rRNA gene sequence showing <98% sequence similarity can be considered as a separate species for this genus. All the 16S rRNA gene sequences used in the analysis were supported by the possession of a signature sequence (5'-tgt gag (a/c)aa tcg cg-3', corresponding to *E. coli* positions 375-388) and a nucleotide bulge (5'-T_n-3', n = 6 or 8; corresponding to a stem-loop structure at *E. coli* positions 200-218) except *Mbr. curvatus* (which instead possess the sequence 5'-ttc tta tgt t-3'). We propose to include the termite gut flagellate symbiont LHD12, the methanogenic endosymbionts of the ciliate *Nyctotherus ovalis,* and rat feces isolate RT reported earlier, as separate species of the genus *Methanobrevibacter*. For organisms that have never been isolated but have been detected in natural samples by rRNA sequence alone (like the majority of sequences in the present study), the ability to estimate *D* will provide a clearer understanding of their genetic and phenotypic diversity.

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A TRANS MECHANISM FOR THE SEQUENTIAL 2'-O-METHYLATION OF ARCHAEAL PRE-TRNATRP NUCLEOTIDES GUIDED BY THE PRE-TRNA'S INTRON-ENCODED BOX C/D RNPS

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RNA processing events to produce mature tRNATrp of Haloferax volcanii, an archaeon, include C/D box-mediated 2'-O-methylation of nucleotides C34 and U39 as well as excision of an intron. Modification of these two residues, located in the 5' (C34) and 3' (U39) exons, respectively, is guided by two box C/D motifs positioned within the pre-tRNA intron. Therefore, it is believed that the two C/D RNP complexes are positioned in cis within the intron when they guide the two ribose-methylation reactions. Folding of pre-tRNA required for the cis methylations and splicing are mutually exclusive and the two cis methylation reactions would require either formation of a pseudoknot or two different structures. Here we show that recombinant archaeal box C/D core proteins from Methanocaldococcus jannaschii, L7Ae, aNop5p and Fibrillarin, form functional C/D RNP complexes with the H. volcanii pre-tRNATrp transcript and methylate both C34 and U39 nucleotides in the pre-tRNA. Efficient nucleotide methylation requires Watson-Crick pairing between the guide and target nucleotides. Site directed mutagenesis of target and guide nucleotides also revealed sequential modification of the targets wherein box C'/D' (internal C/D box) guided U39 methylation first requires box C/D (terminal C/D box) guided C34 methylation. Trans methylations of both pre-tRNAs in the reaction are observed when one pre-tRNA has a mutated target residue and the second pre-tRNA contains the complementary guide residue. In addition, we demonstrate that both circular and linear introns formed during the splicing reaction assemble C/D RNPs and can guide methylation of pre-tRNAs as trans complexes. Finally, analysis of pre-tRNATrp methylation in a cell extract shows that sequential nucleotide modifications were carried out in trans. Previous observations demonstrating the accumulation of excised pre-tRNATrp introns in vivo may therefore suggest that C34 and U39 methylation is guided by the intron-encoded box C/D RNP using a trans mechanism. A trans mechanism would thus eliminate the problem of requiring structural changes within the folded intron to accomplish methylation at both modification sites.

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PROTEOME ANALYSIS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS OF *HALOFERAX VOLCANII* DS2

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Archaea are comprised of different families of microorganisms adapted to environments of habitat characterized by extreme temperature or high salt concentrations. Halophilic archaea are salt-loving microorganisms that can grow optimally at ten times the salinity of seawater (4.5 M NaCl). High-resolution two-dimensional gel electrophoresis (2DE) is an important step in elucidating the global view of proteins present in a cell or tissue at any given time. The recent growth in the area of proteomics has lead to many techniques for isolating and identifying proteins. In halophilic archaea, 2DE analysis is difficult to achieve since high salt concentrations denature and precipitate proteins, thus inhibiting separation of proteins in a 2DE system. Because global proteomic analysis is important in understanding the basic biology of these organisms, and 2DE is a prerequisite for such investigations, a protein isolation protocol that eliminates these difficulties was needed. Using *Haloferax volcanii* DS2 as a model organism, a technique was developed for isolating proteins that yielded 2D gels with minimal streaking and distinct protein spots. This recent study and isolation protocol facilitated the proteomic analysis of *H. volcanii,* resulting in 599 detected protein spots between the pH ranges of 3.5 to 11. This method may also prove beneficial to 2DE analysis of additional halophilic organisms.

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IN VITRO RNP ASSEMBLY AND METHYLATION GUIDE ACTIVITY OF AN UNUSUAL C/D BOX RNA, THE *CIS*-ACTING ARCHAEAL PRE-TRNATRP

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Among the large family of C/D RNAs which guide the site-specific 2'-O-methylation of rRNAs, spliceosomal snRNAs as well as archaeal tRNAs, the intron of euryarchaeal pre-tRNATrp is remarkable. Its pair of antisense elements, together with associated boxes C/D and C'/D', are predicted to guide in *cis*-, instead of *trans*-, two 2'-O-methylations in the tRNA exons. Remarkably, both sites of methylations in the pre-tRNA involve nucleotides within the bulge-helix-bulge (BHB) motif recognized by the archaeal tRNA splicing endonuclease while the RNA-guided methylations and pre-tRNA splicing events depend on mutually exclusive RNA folding patterns. Using the three purified recombinant proteins of archaeal C/D sRNP, L7Ae, Nop5p and aFib, along with a pre-tRNATrp *in vitro* transcript, we have analyzed *in vitro* assembly of a RNP complex and tested its site-specific methylation activity. Recognition by L7Ae of the Kink-turn RNA structural motifs (K-turn) formed by the C/D and C'/D' boxes appears as the basis of the assembly step required for subsequent binding of a Nop5p-aFib heterodimer

at each site. Unexpectedly, even in the absence of L7Ae but at a much higher concentration of Nop5p-aFib, an active RNP complex can still form, probably reflecting the higher propensity of the *cis*-acting system to form guide RNA duplex(es) relative to classical *trans*-acting C/D RNA guides. Moreover, footprinting data of the higher-order RNP complexes, consistent with Nop5p interacting with the non-canonical stem of the K-turn, suggest binding of Nop5p-aFib to the pre-tRNA-L7Ae complex might direct transition from a splicing-competent structure to an RNA conformer displaying the guide RNA duplexes required for site-specific methylation. The archaeal pre-tRNATrp experimental system should provide new insights into the molecular mechanism of C/D snoRNA-guided ribose methylation in eukaryotes.

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CHARACTERIZATION OF THE TWIN-ARGININE TRANSLOCATION PATHWAY OF *HALOFERAX VOLCANII*

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Many archaeal species possess unique adaptations that allow for their survival in extreme environments. In the haloarchaea, evidence suggests that one such adaptation is the routing of nearly all secreted proteins to the twin-arginine translocation (Tat) pathway. The Tat pathway is a recently discovered secretion pathway present in plant chloroplasts and a large number of prokaryotes. Unlike the general secretion pathway, the Tat pathway is able to secrete proteins that have adopted significant tertiary structure prior to translocation. Folding secretory proteins prior to translocation in the cytoplasm may be advantageous in the haloarchaea, as the high intra- and extra-cellular salt conditions may demand cytoplasmic chaperones for the correct folding of the proteins. We have initiated the characterization of the Tat pathway in a model haloarchaeon, Haloferax volcanii. Genetic analyses have revealed that genes encoding the putative Tat machinery proteins TatA2, TatC1, and TatC2 are essential for viability. Interestingly, while a TatA2 paralog, TatA1, is not required for cell growth, overexpression of tatA1 complements a tatA2 deletion. This suggests that the two different TatA paralogs have a similar but distinct function. Cellular fractionation studies suggest that TatA1 and TatA2 localize to both the plasma membrane and the cytoplasm. This may indicate that substrate targeting in the H. volcanii Tat pathway may occur cytoplamically by virtue of a TatA:substrate interaction, as has been suggested for Bacillus subtilis.

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STSV1, A NOVEL SPINDLE-SHAPED VIRUS OF THE HYPERTHERMOPHILIC ARCHAEON *SULFOLOBUS TENGCHONGENSIS*

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In a survey of *Sulfolobus* species and their extrachromosomal genetic elements in acidic hot springs in Tengchong, Yunnan, China, we have isolated and characterized a virus infecting *Sulfolobus tengchongensis*. The virus, denoted STSV1 (*Sulfolobus tengchongensis* spindle-shaped virus 1), is among the largest in known crenarchaeotal viruses. STSV1 has the morphology of a spindle (230×107 nm) with a tail (68 nm in length on average) at one end. Replication of the virus retarded the growth of the host but did not cause the lysis of the host cells. The genome of STSV1 is a 75,294-bp circular double-stranded DNA molecule which is modified in an undetermined fashion. The viral genome has been completely sequenced, and a total of 74 ORFs have been identified. The genome divides into two equal halves of opposite gene orientation: genes in one half of the genome are transcribed predominantly in one direction whereas most of those in the other half in the opposite direction. Putative functions have been assigned to only 11 ORFs. One of these ORFs encodes a putative coat protein, the sole protein detected in virus particles by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. Another ORF encodes a protein with significant sequence similarity to tyrosine integrases. However, no viral integration into the host genome was observed in this study. The products of the rest of the ORFs are probably involved in polysaccharide biosynthesis, nucleotide metabolism and DNA modification. Based on its morphological and genomic features, STSV1 does not appear to belong to any known virus family and is, therefore, a novel virus of *Sulfolobus*.

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EXTREMOPHILES: K-12 SCIENCE CURRICULUM

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Learning science is far more exciting when situated within the natural environment. Montana has many extreme environments (e.g., Yellowstone Park, Glacier Park, alkaline flats, environmental stress areas), and students have always done well in standardized tests on science, attributed to the wide-range of experiences in the rural Montana environment. Extremophiles mark the boundaries and robustness of life, and they are inherently an interesting part of Montana environments. In order to modernize science curriculum, supplement older textbooks, and customize curriculum to the natural environment of Montana, NASA materials related to the Extremophiles theme are used to design grades 5-12 science materials. State standards and benchmarks are identified and essential questions serve as advance organizers for the unit, and used as the controlling device for the development and flow of the unit, lesson by lesson. We developed a novel formulation of a unit

plan through a template that is based on essential questions and offers a range of teaching and learning strategies which promotes different types of thinking required for effective learning. We included a series of inquiry-based activities centered on the search for life on earth and other planets in order to explore the three domains of life and some of the issues that determine the functional diversity of life. Students can investigate extremophile species and their extreme environments and be able to link certain features of extremophiles with their implications for extra-terrestrial life. They also can look at the important role of extremophiles in industry and medicine. In this poster, we will present the unit design and give a full layout of the first lesson. In addition we will give related URL and NASA resources that are used throughout the project.

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USE OF INTACT CELL MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT (MALDI-TOF) MASS SPECTROMETRY FOR THE IDENTIFICATION OF EXTREMOPHILES

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There are few routine methods available to aid in the identification of either archaea or many of the extremophilic bacteria. The use of sequence comparisons of the 16S ribosomal RNA gene is the standard for identifying an organism to genus and species; however this method is generally not sufficient for distinguishing closely related strains. In recent years the use of intact cell MALDI-TOF has been gaining acceptance as a method for identifying bacteria. This method is very simple. One microliter of cell suspension is mixed with a matrix, and then irradiated with a laser in the mass spectrometer. The cell surface molecules are ionized and their masses are registered on a detector, yielding a unique mass spectral profile. We have tested more than 10 genera and 28 spp. of archaea and more than 25 genera and 37 spp. of bacteria and found that all yield unique and reproducible mass spectral profiles. When conspecifics of Halobacterium salinarium and Methanothermobacter thermautotrophicus were compared it was possible to reproducibly distinguish individual strains. The method was sensitive, being able to detect as few as 10(3) cells, although at least 10(5) cells were necessary for a robust identification. Comparisons of different species by UMPGA analysis suggest that the mass spectral profiles are capable of grouping related organisms; however this clustering is not always accurate. We are in the process of evaluating the extent to which mass spectral profiles for individual organism vary as a function of growth phase. We are also testing a large cohort of methanogens to better evaluate MALDI-TOF's ability to routinely identify these organisms. Overall, intact cell MALDI-TOF shows real promise as a universal, rapid and easy method for aiding in the identification of extremophiles.

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TAXONOMIC STUDY OF ALKALIPHILIC BACILLUS STRAINS FOR THE INDUSTRIAL APPLICATIONS

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Alkaliphilic Bacillus species are considerable importance for industrial applications because of their ability to produce some alkaline enzymes such as protease, cyclomaltodextrin glucanotransferase (CGTase) and cellulase(1). These enzymes produce extracellular that are resistant to high pH and/or high temperature conditions. Since Vedder (2) first isolated the aerobic, endospore forming, obligate alkliphilic bacteria Bacillus alcalophilus, many obligate or facultative alkaliphilic Bacillus have been isolated for industry, biotechnology and physiology. And more than 18 species of alkaliphilic Bacillus spp., have been identified to date (3, 4). However, most of these bacteria were still left behind by untouched or not identified up to the species level. In this study, 28 alkaliphilic strains were selected from those used in enzymes studies by Horikoshi and coworker. The strains were subjected to a polyphasic taxonomic study, including 16S rRNA sequence, genomic DNA G+C content, DNA-DNA hybridizations, fatty acid analysis, morphological characterization and biochemical characterization. By comparing the groupings obtained by genomic DNA G+C content and the construction of a phylogenetic tree on the basis of the 16S rRNA sequence, twelve clusters of similar strains could be recognized. DNA-DNA hybridization revealed that these clusters represented eight novel genospecies. Based on phenotypic characteristics supported six novel species in the genus Bacillus.

1. Horikoshi, K. (1999) Alkaliphiles. Kodansha, Tokyo
2. Vedder, A. (1934). Antonie Leeuwenhoek J Microbiol 1, 143-147.
3. Nielsen, P., Fritze, D. & Priest, F. G. (1995). Microbiology 141, 1745-1761.
4. Yumoto, I., Yamaga, S., Sogabe, Y., Nodasaka, Y., Matsuyama, H., Nakajima, K. & Suemori, A. (2003). Int J syst Evol Microbiol 53, 1531-1536.

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CHARACTERIZATION OF A NOVEL MEMBER OF THE ORDER THERMOPLASMATALES ISOLATED FROM A SOLFATARIC FIELD OF HAKONE, JAPAN

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The order Thermoplasmatales comprises extremely acidophilic, moderately-thermophilic or mesophilic euryarchaeotes represented by three genera Thermoplasma, Picrophilus and Ferroplasma. Recently, we isolated a novel member of the order Thermoplasmatales by applying the MPN/PCR method from a solfataric field of Hakone, Kanagawa, Japan. In this study, we describe characteristics of the novel strain, IC-189, to elucidate the taxonomic position. This organism was strictly aerobic, and grew

heterotrophically on yeast extract. The optimal temperature and pH for growth were 60C and pH 3.0 respectively, although growth was observed in the temperature range between 38 and 68C and the pH range between 1.0 and 5.0. Cells of the isolate were irregular cocci, highly variable in size ranging 1.0 to 8.0 µm in diameter. It did not show motility or flagellum. On gelrite-based plate, it formed "fried egg"-like colonies of variable size. Except for glucose and mannose, none of the sugars, amino acids, organic acids or other organic compounds tested were utilized as carbon and energy source. Addition of ampicillin, erythromycin, novobiocin, penicillin, rifampicin and vancomycin (100µg/ml each) inhibited growth. The core lipid consisted mainly of tetraether lipid. Major quinone isoprenologue was MK-7. The DNA base composition of the strain was 56.1 mol%G+C. Phylogenetic analysis based on the 16S rDNA sequences showed that the strain was incorporated into the order Thermoplasmatales, however, it was distantly related to the known genera (i. e. Thermoplasma, Picrophilus and Ferroplasma) with sequence similarities of 80.0-91.5%. On the basis of the phenotypic, phylogenetic properties described above, strain IC-189 should represent a new genus and species in the order Thermoplasmatales.

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MOLECULAR CHARACTERIZATION OF A NOVEL GLYCOSYLTRANSFERASE FROM *PYROCOCCUS HORIKOSHII*

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The gene of novel enzyme having glycosyl-transferring activity from *Pyrococcus horikoshii* was cloned using PCR with pET 30 vector. The open reading frame of this gene, which has not been recognized so far, consists of 1,250 nucleotides and encodes 416 amino acids. The enzyme was then constitutively expressed in *E. coli* using p6xHis119 vector and the gene product with N-terminal six histidine tag was purified to homogeneity by Ni-NTA affinity chromatography. The molecular weight was determined to be 49.8 kD by SDS-PAGE and MALDI-TOF mass spectrometry. The enzyme had an optimum pH of 5.5 in the activity, and it was relatively stable at pH ranging from 6 to 8. Unexpectedly, the enzyme did not show any significant thermal stability, even though derived from the hyperthermophile,. The optimum temperature was found to be 40°C, and the stability was sharply decreased above 40°C. When the deduced amino acid sequence of the enzyme was compared with those of another proteins available in terms of primary and tertiary structures, there was predicted to belong to glycosyltransferase group 1 family. Interestingly, the enzyme could employ various maltooligosaccharides larger than maltose as both donor and acceptor to produce glucose and á-1,4-linked disproportionated products of the substrate used. The results implied that the enzyme follows an action pattern of 4-á-glucanosyltransferase, which catalyzes the disproportionation of maltooligosaccharide by the transfer of the glucanosyl unit to a new C-4 position in another one. In addition, the enzyme showed a degrading activity on various kinds of starches, resulting in the alteration of the side chain distribution in the amylopectin through intermolecular rearrangement. These results suggested that the enzyme could be utilized to produce various DPs of maltooligomeric dextrins and structured-and-modified starch.

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TUNGSTEN-TRANSPORT AND CELLULAR DISTRIBUTION IN *PYROBACULUM AEROPHILUM*

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Pyrobaculum aerophilum is a hyperthermophilic archaeon that can generate its energy either by oxygen respiration under microaerophilic conditions or anaerobically by nitrate respiration (1, 2). We have previously shown that growth of *P. aerophilum* with nitrate is strictly dependent on the presence of tungstate in the culture medium (2). Tungsten is the heaviest metal found in biological systems and often replaces molybdenum as a cofactor in enzymes of bacteria and archaea that are thriving in a high tungsten environment (3). In previous studies we have established that nitrate reductase activity of P. aerophilum decreases 4-fold when the tungstate concentration is increased above a 1ìM threshold (2). A highly active molybdenum-containing nitrate reductase (Nar) was purified from low-tungsten-grown cells (4). We recently isolated a tungsten-containing nitrate reductase from cells that were grown with optimal tungsten concentration. The specific activity of the tungsten-Nar is about 2-fold lower than that of the molybdenum-Nar. We report here that *P. aerophilum* contains two energy-requiring tungstate transport systems. A high and a low affinity transport system is present in low tungsten grown cells. In contrast, *P. aerophilum* cultured under optimal tungsten concentration exhibits only a low affinity transport.

1. Völkl, P., R. Huber, E. Drobner, R. Rachel, S. Burggraf, A. Trincone, and K. O. Stetter. 1993. *Pyrobaculum aerophilum* sp. nov., a novel nitrate-reducing hyperthermophilic archaeum. Appl. Environ. Microbiol. 59:2918-2926.
2. Afshar, S., C. Kim, H. G. Monbouquette, and I. Schröder (1998) Effect of tungstate on nitrate reduction by the hyperthermophilic Archaeon *Pyrobaculum aerophilum*. Appl. Environment. Microbiol. *64*:3004-3008.
3. Kletzin, A., and M. W. W. Adams. 1996. Tungsten in biological systems. FEMS Microbiol. Rev. 18:5-63.Afashar
4. Afshar, S., E. Johnson, S. de Vries, and I. Schröder (2001) Properties of a thermostable nitrate reductase from the hyperthermophilic Archaeon *Pyrobaculum aerophilum*. J. Bacteriol. 183:5491-5495

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A SIMPLE METHOD FOR DETERMINING BIOMASS OF METHANOGENS

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Neubauer cell counting chamber method is modified to measure the number of nonmethanogenic and methanogenic bacteria of microbial methanogenic communities from thermophilic, psychrophilic as well as mesophilic environments using the area- or volume-based quantification. Total number of bacteria is measured by examining the slide with the phase-contrast microscope. Whereas number of epifluorescent bacteria are counted by examining the slide with UV-fluorescence microscope equipped with an excitation filter 330-380 and a barrier filter BA 435. The counts of bacteria are restricted to a limited volume of a square (0.1 mm each side and 0.1 mm depth) formed of small four squares having each side of 0.05 mm and then expressed in counts per ml. Counts of epifluorescent cells are also restricted to the volume of a right circular cylinder apparently formed or observed, by adjusting the diaphragm of UV-source, in the small four squares with a diameter of 0.1 mm. The volume of this right circular cylinder will be " r^2h = 3.14 x 0.05 x 0.05 x 0.1 mm. The enumerated counts of epifluorescent cells are then mathematically calculated and multiplied by a correction factor of 1.274 to get the counts of epifluorescent cells per ml volume. Pure cultures of *Methanococcoides methylutens*, *Methanobacterium formicicum* and *Methanobacterium bryanti* showed 100, 96.3 and 93 percent accuracy respectively in enumeration of epifluorescent cells when compared to that of phase-contrast. For segregation of clumps of *Methanosarcina* cells, the samples collected from different environments were treated with 3 M NaCl and then the cells were enumerated. Clumps of *Methanosarcina* were not so rigid as compared to that of laboratory grown cells of *Methanosarcina* where most favorable conditions were maintained, therefore cells of *Methanosarcina* from different environments were easily segregated by salt treatment.

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RESCUE OF PROTEIN SPLICING ACTIVITY FROM A MAGNETOSPIRILLUM MAGNETOTACTICUM INTEIN-LIKE ELEMENT

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The self-catalytic protein splicing mechanism is mediated by an intein plus the first amino acid following the intein C-terminus (termed the +1 residue). In all inteins studied to date, the +1 residue is a Cysteine, Serine or Threonine (InBase: <http://www.neb.com/neb/inteins.html>). This residue is an essential nucleophile in two steps of the protein splicing pathway: ligation of the extein fragments and conversion of the (thio)ester bond between the exteins to a peptide bond. An intein-like element in a hypothetical protein (gene Magn8951) from Magnetospirillum magnetotacticum strain MS-1 has all the intein signature sequences except at the +1 position, where it has a Tyr. Although the Tyr side-chain hydroxyl group can potentially perform a transesterification reaction to ligate the exteins, Tyr has never been shown to undergo an acyl rearrangement analogous to Ser, Thr or Cys, which is needed to form a peptide bond between the ligated exteins. If it spliced, this intein-like element would require a third protein-splicing mechanism. The Magn8951 intein-like element with varying lengths of native extein sequence was cloned into a model fusion protein. Splicing and cleavage activity of the expressed fusion protein was analysed on Coomassie blue stained protein gels and Western blots. Protein splicing was not observed and N-terminal cleavage predominated. Mutation of Tyr+1 to Phe or Ala demonstrated that the Tyr side-chain hydroxyl is not necessary for N-terminal cleavage. Protein splicing activity could be rescued by 'reversion' of Tyr+1 to Cys. These results suggest that the Magn8951 intein-like element is an intein that lost the ability to splice when its +1 residue was mutated to Tyr. Why hasn't this element accumulated more mutations and become a pseudo-intein? One possibility is that this is a recent mutation. Another possibility proposed by Pietrokovski and co-workers for bacterial intein-like elements (BIL) is that the host protein may be functional after N-terminal cleavage releases the intein-like element (Amitai et al., Mol Microbiol. 2003 47:61-73). In the case of Magn8951, N-terminal cleavage may result in an active product especially since its C-extein is only 24 amino acids long.

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RESTRICTION-MODIFICATION SYSTEMS IN A HOT SPRING MICROBIAL COMMUNITY

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Restriction-modification systems are widespread among prokaryotes, including both eubacteria and archaea. Methyltransferases are easy to identify by their signature motifs. However, restriction endonucleases lack definitive signature sequences, but are usually adjacent to their partner methyltransferases. Sequenced genomes reveal that on average there are four or five putative DNA methyltransferases per genome. We are attempting to access this broad diversity of restriction-modification in a culture-independent manner. A library prepared from total DNA isolated from a Yellowstone National Park hot spring was examined for the presence of restriction modification systems. The hot spring for this study was located in the White Creek basin of YNP. The source pool was 0.5 meters across, emitting water at 88°C, pH 8.5. The sample studied consisted of a mixture of pink, orange, tan and green filaments growing in the small effluent channel of the spring at approximately 70° to 75°C. DNA was purified from 1.5ml of packed filaments and a plasmid library containing approximately 1,000,000 individuals was generated. The methylase selection method was employed to see if several commonly occurring restriction systems might be represented in this library. The endonucleases HaeIII (recognizing 5'-GGCC-3'), MseI (recognizing 5'-TTAA-3'), HinfI (recognizing 5'-GANTC-3') and TaqI (recognizing 5'-TCGA-3') were used. Plasmid DNAs conferring resistance to each of these endonucleases were identified. Four clones were sequenced (one for HaeIII, one for

HinfI and two for TaqI resistant DNAs). The two TaqI resistant clones were quite different. One methyltransferase was next to an inactive (two frameshifts) copy of TaqI endonuclease. The other methyltransferase had no endonuclease next to it. The two methyltransferases were only 62% identical at the protein level. These results confirm that restriction modification systems are present in the bacteria and/or archaea inhabiting this thermal environment in Yellowstone National Park. The enzymes identified in this initial screen will be further characterized and compared to previously known examples.

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A SYSTEM FOR INDUCIBLE OVEREXPRESSION OF RECOMBINANT PROTEIN IN THE ARCHAEON *METHANOSARCINA ACETIVORANS*

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Large quantities of protein are generally required for biochemical and structural analyses. Due to the difficulty in mass culturing many extremophilic Archaea, these proteins are often obtained by recombinant overexpression in bacterial systems. However, some proteins expressed in these systems fail to exhibit activity, probably due to differences in post-translational processing between the domains. To circumvent these problems, an overexpression system was developed for *Methanosarcina acetivorans*. This organism was chosen because it is a mesophilic archaeon that can be mass cultured to high densities in a bioreactor and it has an efficient genetic system. The overexpression system utilizes a shuttle vector that propagates in both *M. acetivorans* and *Escherichia coli* and contains the promoter for the *Methanosarcina thermophila* carbon monoxide dehydrogenase/acetyl coenzyme A synthase operon, which is highly regulated in *M. acetivorans* in response to growth substrate. The activity of the promoter, which is high when the organism is grown on acetate, is attenuated by adding different concentrations of the methylotrophic substrates methanol or trimethylamine. A multiple cloning site flanked by coding sequence for either N- or C-terminal 6-histidine polypeptide (6xHis) fusions is located downstream from the promoter. The gene of a target protein can be cloned into the multiple cloning site of the plasmid and expressed in its native form or with an N- or C-terminal 6xHis fusion in *M. acetivorans*. To test the system, the gene for a *Methanocaldococcus jannaschii* prolyl tRNA synthetase (ProRS) was cloned into the multiple cloning site of the overexpression plasmid and expressed with an N-terminal 6xHis fusion in *M. acetivorans*. The recombinant strain of *M. acetivorans* was grown with acetate in a 14-liter bioreactor configured to run as a pH auxostat. ProRS was successfully purified from the harvested cells using immobilized metal affinity chromatography. This work demonstrates the ability to express a protein from a hyperthermophilic archaeon in *M. acetivorans* for scale-up and subsequent purification of the protein. By utilizing this overexpression system, it may be possible to characterize archaeal proteins that cannot be actively expressed in currently available systems.

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DEINOCOCCUS RADIODURANS: GAMMA-RADIATION RESISTANCE WITHOUT RINGLIKE NUCLEOIDS

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Deinococcus radiodurans is extremely resistant to ionizing radiation. Electron microscopy studies do not support the hypothesis that ringlike nucleoids (RNs) of *D. radiodurans* are needed for recovery from irradiation. *D. radiodurans* has been reported to grow predominantly as diplococci in defined minimal medium (DMM), even in the late stages of growth. We examined cells grown in DMM or undefined rich medium (TGY) by electron microscopy to determine the prevalence of RNs, and also tested cells for their resistance to ionizing radiation (IR). The resistance of late-log phase cultures grown in TGY, that contained cells that lacked RNs, was greater than early-stationary phase cultures grown in DMM, that contained cells with RNs. *Deinococcus grandis* grows as single cells, displays similar resistance to IR in either TGY or DMM medium, and cells rarely displayed nuclear structures that could be described as ringlike. So far, neither genomic nor experimental analyses unequivocally support any model that explains the extreme radioresistance of *Deinococcaceae*.

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