FINAL PROGRESS REPORT
DE-FG02-93ER20117
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The Characterization of Psychrophilic Microorganisms and their potentially useful Cold-Active Glycosidases

SUMMARY OF RESULTS SINCE THE 2007 PROGRESS REPORT.

Our studies of novel, cold-loving microorganisms have focused on two distinct extreme environments. The first is an ice core sample from a 120,000 year old Greenland glacier. The results of this study are particularly exciting and have been highlighted with press releases and additional coverage. The first press release in 2004 was based on our presentation at the General Meeting of the American Society for Microbiology and was augmented by coverage of our publication (Appl. Environ. Microbiol. 2005. Vol. 71:7806) in the Current Topics section of the ASM news journal, "Microbe." Of special interest for this report was the isolation of numerous, phylogenetically distinct and potentially novel ultrasmall microorganisms. The detection and isolation of members of the ultrasmall population is significant because these cells pass through 0.2 micron pore filters that are generally used to trap microorganisms from environmental samples. Thus, analyses by other investigators that examined only cells captured on the filters would have missed a significant portion of this population. Only a few ultrasmall isolates had been obtained prior to our examination of the ice core samples. Our development of a filtration enrichment and subsequent cultivation of these organisms has added extensively to the collection of, and knowledge about, this important population in the microbial world.

A unique aspect of our isolate examination was the survey of plasmids and antibiotic sensitivities and resistances. One of our *Arthrobacter*-related isolate contained a small plasmid which we isolated, sequenced, and used as the basis for constructing a shuttle vector. We then developed an electroporation-transformation protocol and demonstrated successful transfer of the vector into a variety of Gram-positive isolates. This vector could be useful in a wide array of genetic studies and the results were published in the journal "Extremophiles" and the abstract is added below.

Our recent characterization of these ultrasmall celled isolates led to additional manuscripts and our 2008 abstract for the American Society for Microbiology which they selected for a press release (copied below) and it continues to be cited in a variety of news and internet articles. Abstracts from the publications of these results are included in this report and the papers are supplied as attached PDFs. Additional manuscripts describing new ultrasmall celled isolates that represent new species are either in revision for publication or in preparation for submission.

The second extreme environment examined was sediment from the deep sea subsurface. Even though calculations suggest that the deep subsurface contains a high percentage of the Earth's biomass, we know very little about the microorganisms inhabiting this high-pressure environment. We have obtained sediment cores from the Ocean Drilling program and have investigated several isolates and the publication "Biddle, House, and Brenchley. 2005. Jørgensen, B.B., D'Hondt, S.L., Miller, D.J., et al., 2005 (Eds.) *Proceedings of the Ocean Drilling Program, Scientific Results* Volume 201" described results with isolates obtained

following an enrichment for heterotrophy and the molecular analysis of 16S rDNA extracted from samples. In addition, a collaborative examination of the deep sediment cores showed that archaeal cells actually outnumber the bacterial cells. This surprising finding was published in PNAS (Biddle, J. et al. 2006. Novel heterotrophic *Archaea* dominate sedimentary subsurface ecosystems off Peru. Proc. Natl. Acad. Sci. USA, 103:3846-3851.). Surprisingly, the FISH coupled with SIMS results suggested that the archaeal cells are not using the methane as a carbon source in this environment and raises the question of whether these cells have a unique metabolism for methane oxidation.

The most recent examination of the subsurface microorganisms was a metagenomic analysis of DNA extracted from different sediment samples. A metagenomic analysis was made using whole genome amplification and pyrosequencing of DNA extracted from sediments to further explore the microbial diversity and overall community composition within this environment. The 16S small subunit ribosomal gene analyses also suggest that Crenarchaeota are abundant and quantitative PCR confirms that uncultivated Crenarchaeota are indeed a major microbial group in these subsurface samples. These findings show that the marine subsurface is a distinct microbial habitat and is different than environments previously studied by metagenomics. (Abstract for PNAS article is given below and this was selected for a press release from Penn State.)

ABSTRACTS FROM RECENT PUBLICATIONS

1. Miteva V.I., Sowers T.A., Brenchley J.E, (2007) Production of N_2O by ammonia oxidizing bacteria at subfreezing temperatures as a model for assessing the N_2O anomalies in the Vostok ice core. Geomicrobiology Journal, 24: 451-459.

It was suggested that the abnormally high N_2O values found in 130,000–160,000 year-old Vostok ice core samples, characterized by high δ15N and low δ18O values, resulted from in situ microbial N2O production. To substantiate these observations we obtained new geochemical data from the last glacial period and showed the existence of additional small N_2O anomalies. To test the hypothesis that microbial metabolism could contribute to these anomalies, we developed protocols for examining the ability of *Nitrosomonas cryotolerans* cells to produce N_2O at subfreezing temperatures. Our results show that these model, frozen cultures produce N_2O at temperatures as low as −32◦C.

2. Miteva, V., S. Lantz, and J. Brenchley. 2008. Characterization of a cryptic plasmid from a Greenland ice core *Arthrobacter* isolate and construction of a shuttle vector that replicates in psychrophilic high G+C Gram-positive recipients. Extremophiles 12: 441-449.

Over 60 Greenland glacial isolates were screened for plasmids and antibiotic resistance/ sensitivity as the first step in establishing a genetic system. Sequence analysis of a small, cryptic, 1950 bp plasmid, p54, from isolate GIC54, related to *Arthrobacter agilis,* showed a region similar to that found in theta replicating *Rhodococcus* plasmids. A 6002 bp shuttle vector, pSVJ21, was constructed by ligating p54 and pUC18 and inserting a CAT cassette conferring chloramphenicol resistance. Candidate Gram-positive recipients were chosen among glacial isolates based on phylogenetic relatedness, relatively short doubling times at low temperatures, sensitivity to antibiotics, and absence of indigenous plasmids. We developed an electroporation protocol and transformed seven isolates related to members of the *Arthrobacter*, *Microbacterium*, *Curtobacterium*, and

Rhodoglobus **genera with pSVJ21. Plasmid stability was demonstrated by successive transformation into *Escherichia coli* and four Gram-positive isolates, growth without antibiotic, and plasmid re-isolation. This shuttle vector and our transformation protocol provide the basis for genetic experiments with different high G+C Gram-positive hosts to study cold adaptation and expression of cold-active enzymes at low temperatures.**

3. Loveland-Curtze J., Miteva V., Brenchley J. (2008) *Herminiimonas glaciei* sp. `nov., a novel ultramicrobacterium from 3042 m deep Greenland glacial ice. (under revision for International Journal of Systematic and Evolutionary Microbiology).

A gram-negative ultramicrobacterium (strain UMB49T) was isolated from a 120,000-year-old, 3042 m deep Greenland glacier ice core using a 0.2 µm filtration enrichment procedure. Phylogenetic analysis of the 16S rRNA gene sequence indicated that this strain belongs to the genus *Herminiimonas* of the family *Oxalobacteraceae* of the *Betaproteobacteria*. It is most closely related to *H. saxobsidens* (99.6 %), *H. arsenicoxydans (*98.4 %), *H. aquatilis* (97.6 %), and *H. fonticola* (97.6 %). Cells were small thin rods with an average volume of 0.043 µ3 and possessed 1-2 polar and/or 1-3 lateral very long flagella. Original colony pigmentation was brown-purple but after re-cultivation the colonies were translucent white to tan coloured. The strain grew aerobically but tolerated less oxygen. UMB49T produced catalase and oxidase but did not reduce nitrate. Sole carbon sources included citrate, succinate, malate, lactate and alanine. The strain produced acid from L-arabinose, D-arabinose, L-xylose, D-xylose and D-ribose. The DNA G+C content is 59.0 mol%. Based on the differences from the validated *Herminiimonas* species, we concluded that the strain represents a novel species for which the name *Herminiimonas glaciei* sp. nov. is proposed.

4. Abstract for Biddle, J., Fitz-Gibbon, S , Schuster, S. Brenchley. J and House, C. 2008. Metagenomic signatures of the Peru Margin subseafloor biosphere. (Proc. Natl. Acad. In Press. Will appear online between 7-21 and 7-25)

The subseafloor marine biosphere may be one of the largest reservoirs of microbial biomass on Earth and has recently been the subject of debate in terms of the composition of its microbial inhabitants, particularly on sediments from the Peru Margin. A metagenomic analysis was made using whole genome amplification and pyrosequencing of sediments from Ocean Drilling Program Site 1229 on the Peru Margin to further explore the microbial diversity and overall community composition within this environment. A total of 61.9 Megabases of genetic material was sequenced from sediments at horizons 1, 16, 32 and 50 meters below seafloor. These depths include sediments from both primarily sulfate-reducing and also methane- generating regions of the sediment column. Many genes of the annotated genes, including those encoding ribosomal proteins, corresponded to those from the Chloroflexi and Euryarchaeota. However, analysis of the 16S small subunit ribosomal genes suggests that Crenarchaeota are the abundant microbial member. Quantitative PCR confirms that uncultivated Crenarchaeota are indeed a major microbial group in these subsurface samples. These findings show that the marine subsurface is a distinct microbial habitat and is different than environments previously studied by metagenomics, especially due to the predominance of uncultivated archaeal groups.

5. Abstract from the ASM Meeting from which the press release was derived and which will be the subject of a future manuscript. **Loveland-Curtze, J.,** V. Miteva, and J. Brenchley. 2008. Novel Ultramicrobacterial Isolates from a Deep Greenland Ice Core Represent a Proposed New Species, *Chryseobacterium greenlandensis* sp. nov. American Society for Microbiology General Meeting 2008.

Three orange, Gram-negative, aerobic bacterial isolates, designated UMB 10, UMB 14 and UMB 34, were obtained from an enrichment culture inoculated with filtrate from a melted 3042 m deep Greenland ice core sample passed through a 0.2 um filter. Analysis of 16S rRNA gene sequences showed that the three isolates belonged to a single species within the genus *Chryseobacterium*. The closest phylogenetic neighbors among the 29 validly described *Chryseobacterium* species were *C. soldanellicola* (2.7%), *C. indoltheticum* (3.3%), *C. scophthalmum* (3.3%), *C. balustinum* (3.3%) and *C. piscium* (3.9%), which formed a stable cluster with the isolates. Electron micrographs showed that the cells were short rods, had cellular volumes within the range of

ultramicrobacteria (<0.1 um^3), lacked flagella, formed small buds, and produced copious extracellular material. Growth was observed from 2^{o}C to 37^{o}C with an optimal growth rate (doubling time 2 h) at 30^{o}C in TSB. Colonies were convex, shiny, orange on TSA and yellow on R2A. The flexirubin reaction typical for *Chryseobacterium* was positive. Cells were oxidase and catalase positive. The two major fatty acids in cells grown at 25^{o}C were 15:0 iso (38.4%) and 17:0 iso 3OH (21.8%). On the basis of these results, the isolates represent a new species of the genus *Chryseobacterium*, for which the name *Chryseobacterium greenlandensis* sp. nov. is proposed. The type strain is UMB 34^T.

PRESS RELEASE FOR THE AMERICAN SOCIETY FOR MICROBIOLOGY MEETING

Survivor Greenland: A Novel Bacterial Species is Found Trapped in 120,000-Year-Old Ice

BOSTON – June 3, 2008

A team of Penn State scientists has discovered a new species of bacteria that survived for more than 120,000 years within Greenland glacier ice. This new species also represents the largely uncultivated, ultra-small microbes dominating many environmental populations. The microorganism's ability to persist in the low temperature, high pressure, low oxygen, and dilute nutrients of the glacier makes it particularly useful for studying ways life endures stresses found in other extreme environments on Earth and elsewhere in the solar system.

This new species is only one of about 10 that have been described originating from polar ice and glaciers and is among the ubiquitous, yet mysterious, ultra-small bacteria. Although microbial cells by definition are small, a typical *Escherichia coli* cell would completely dwarf these diminutive cells at one-tenth or one-twentieth its volume. These tiny cells pass through filters used to trap microbes from water and soil samples and were overlooked in studies of cells collected on the filters.

In addition, ultra-small cells could be unknown contaminants in media and medical solutions "sterilized" using these filters. The researchers actually used this filterability trait to enrich for ultra-small cells by saving and incubating the melted ice filtrate at low temperature in nutrient depleted, anaerobic media. Three of the ultra-small isolates obtained were genetically related to known bacteria in the genus *Chryseobacterium* found in fish, roots, and marine mud. The proposed name for this novel species is *Chryseobacterium greenlandensis* sp. nov. The description of this one species is a significant step in the overall endeavor to discover, cultivate, and use the distinctive features held by these unknown members of the microbial world.

The work was conducted by three members of the Penn State Department of Biochemistry and Molecular Biology: Jennifer Loveland-Curtze, senior research associate, Vanya I. Miteva, senior research associate, and Jean E. Brenchley, professor. This research was supported by the United States Department of Energy, the National Aeronautics and Space Administration (NASA), and the National Science Foundation. The results will be presented at the 108th American Society for Microbiology General Meeting in Boston, Massachusetts on 3 June 2008 at 10:30 a.m. (Session 142/N, Extreme Environments - I, poster N-156).

Cultivation of previously undiscovered microbes and the description of new species are critical for understanding the wide array of features exhibited by diverse organisms. Microbes comprise up to one-third or more of the Earth's biomass, yet fewer than 8,000 species of the potential 3,000,000 have been described. To further study *Chryseobacterium greenlandensis* sp. nov., the researchers characterized the species' genetic, physiological, biochemical, and morphological features. The type strain of *C. greenlandensis*, UMB34, shares many traits with the other members of the *Chryseobacterium* genus, but also exhibits differences in carbohydrate utilization, enzyme production and growth.

It has unique morphological features not observed in other *Chryseobacterium* species. Its cells form small buds and large quantities of extracellular material. *Chryseobacterium* species are aerobes, as is UMB34, but it also tolerates a

reduced oxygen environment similar to the conditions 3 km down in the Greenland ice. The team hopes that by continuing to examine this species, as well as others living in the Greenland ice, they can learn how cells survive and discover isolates with additional fascinating and useful properties.

REFERENCES FOR PUBLICATIONS AND ABSTRACTS RESULTING FROM THIS GRANT
(Since 2004)

Biddle, J., Fitz-Gibbon, S , Schuster, S. Brenchley. J and House, C. 2008. Metagenomic signatures of the subseafloor biosphere (Proc. Natl. Acad. In Press)

Loveland-Curtze J., Miteva V., Brenchley J. (2008) *Herminiimonas glaciei* sp. `nov., a novel ultramicrobacterium from 3042 m deep Greenland glacial ice. (under revision for International Journal of Systematic and Evolutionary Microbiology).

Miteva, V., S. Lantz, and J. Brenchley. 2008. Characterization of a cryptic plasmid from a Greenland ice core *Arthrobacter* isolate and construction of a shuttle vector that replicates in psychrophilic high G+C Gram-positive recipients. Extremophiles 12: 441-449.

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**Loveland-Curtze J., Miteva V., Brenchley J. (2008) Novel Ultramicrobacterial isolates from a deep Greenland ice core represent a proposed new species, *Chryseobacterium greenlandensis* sp. nov. 108 ASM General Meeting, Boston, MA.

Miteva V.I., Sowers T.A., Brenchley J.E, (2007) Production of N_2O by ammonia oxidizing bacteria at subfreezing temperatures as a model for assessing the N_2O anomalies in the Vostok ice core. Geomicrobiology Journal, 24: 451-459.

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Miteva, V., S. Lantz, and J. Brenchley. 2006. Construction of a new plasmid shuttle vector for psychrophilic high G+C Gram positive bacteria. 106th Abst. Gen. Mtg. ASM. I-093.

Lowit, M., V. Miteva, J. Brenchley, and M. Voytek. 2006. Assessment of three methods of whole genome amplification: application for environmental samples with low microbial biomass. 106th Abst. Gen. Mtg. ASM. Q-433.

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Miteva, V. and J. Brenchley. 2006. Phylogenetic and physiological studies of a diverse bacterial and archaeal population from a deep Greenland glacier ice. Abst. International Conf. on Alpine and Polar Microbiology, Innsbruck Austria.

Sowers, T., V. Miteva, and J. Brenchley. 2006. Assessing N_2O anomalies in the Vostok ice core in terms of in-situ N_2O production by nitrifying microorganisms. Abst. International Conf. on Alpine and Polar Microbiology, Innsbruck Austria.

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Biddle, J., C. House and J. E. Brenchley . 2005. Enrichment and cultivation of microorganisms from deep-sea sediment Peru Trench (ODP Site 1230). In: Proceedings of the Ocean Drilling Program, Scientific Results Jorgensen, B., S. D'Hondt, D. Miller, et al., (eds) 201:1-19 (http://www-odp.tamu.edu/publications/201_SR/107/107.htm).

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**Miteva, V. and J. Brenchley. 2005. Detection and isolation of ultrasmall microorganisms from a 120,000 year old Greenland glacier ice core. Appl. Environ. Microbiol. 71:7806-7818.

Miteva, V. I., P. Sheridan, and J. Brenchley. 2004. Phylogenetic and physiological diversity of microorganisms isolated from a deep Greenland glacier ice core. Appl. Environ. Microbiol. 70:202–213.

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Miteva, V. and J. Brenchley. 2004. Diversity of ultra-small microorganisms in Greenland glacier ice. Abstracts for ASM Conference on Extremophiles.

Coker, J. and J. Brenchley. 2004. Characterization of wild-type and mutant forms of a cold-active beta-galactosidase from an antarctic *Arthrobacter* isolate provides insight into cold-activity. Abstracts for ASM Conference on Extremophiles.

ADDITIONAL PUBLICATIONS AND PRESENTATIONS FROM MY PROGRAM:

Miteva V.I. Microbiology of ancient Greenland ice. 107 ASM General Meeting, Toronto, May 2007 (invited presentation).

Miteva V.I. (2008) Bacteria in snow and glacier ice. Book chapter in: Psychrophiles: from biodiversity to biotechnology, R. Margesin, F. Schinner, J.-C. Marx, C. Gerday (eds), Springer-Verlad Berlin Heidelberg, pp.31-50.

FOOTNOTES:

*****Paper was selected by ASM News for their Journal Highlights section (ASM News:60#3, 1994)

******Presentations were selected by ASM for a press release and received extensive general publicity.

*******Paper was selected by ASM for coverage in Current Topics Section of Microbe (formally ASM News) (2006.1:57-58).

******** Paper was selected by ASM for coverage in Current Topics Section of Microbe (2006. 1:216-217).