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The International Symposium on Cellular and Molecular Biology of Phosphate and Phosphorylated Compounds in Microorganisms, Woods Hole, Massachusetts, 12–17 September 1993, is sponsored by:

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Annamaria Torriani, for the Organizing Committee

SYMPOSIUM PROGRAM

International Symposium on Cellular and Molecular Biology of Phosphate and Phosphorylated Compounds in Microorganisms
12–17 Sept. 1993 at Woods Hole, Mass., USA

NOTE: Times shown in italics (*7:30*) are A.M., all others P.M.

Registration: Sunday, 2:00–6:00; Monday, *7:00–8:45*, 2:00–4:00
Sunday: Reception, 6:00–7:00; Dinner, 7:00–9:00
Monday–Friday: breakfast, *7:00–8:30*

Session I. Regulation of Phosphate Metabolism in Bacteria MONDAY 13th

8:45–9:00 CONVENERS: Annamaria Torriani-Gorini (USA) and Ezra Yagil (Israel)

9:00–9:30 Naryana N. Rao (Dept. Biochem., Stanford Univ. Sch. Med., USA)
"Phosphate, phosphorylated metabolites and Pho regulon"

9:30–10:00 Kozo Makino (Res. Inst. for Microbial Diseases, Osaka Univ., Japan)
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"Multiple controls of the *Escherichia coli* Pho regulon by the P_i sensor PhoR, the catabolite regulatory sensor CreC, and acetyl phosphate"

10:30–10:45 (break)

10:45–11:15 Graeme B. Cox (John Curtin Sch. Med. Res., Australian Nat. Univ., Australia)
"The molecular mechanism of phosphate movement through the Pst System, an ABC transporter"

11:15–11:45 Winfried Boos (Dept. Biology, Univ. Konstanz, Germany)
"The *E. coli* Ugp transport system is regulated in its activity by internal phosphate"

11:45–12:15 F. Marion Hulett (Dept. Biol. Sci., Univ. Illinois–Chicago, USA)
"*B. subtilis* alkaline phosphatases controlled by sequential action of genetic switches"

Lunch, 12:30–2:00

2:30–4:30 **Round Table. Structure-Function of Alkaline Phosphatase**
Evan Kantrowitz, Moderator (Dept. Chemistry, Boston College, USA)
Cathy Brennan (Abbott Labs., Abbott Park, Ill., USA)
Joseph E. Coleman (Dept. Molec. Biophys. & Biochem., Yale Univ., USA)
Eunice Kim (Vertex Pharmaceuticals, Cambridge, Mass., USA)
Albert Matlin (Dept. Chemistry, Oberlin College, USA)
Harold W. Wyckoff (Dept. Molec. Biophys. & Biochem., Yale Univ., USA)

Cocktails, Dinner, 5:30–7:00

Session II. Regulation of Phosphate Metabolism in Yeast MONDAY 13th

7:00–7:15 CONVENERS: Yasuji Oshima (Japan) and Harlyn Halvorson (USA)

7:15–7:45 Yasuji Oshima (Dept. Biotechnol., Osaka Univ., Japan)
"The regulatory circuit for the phosphatase genes in *Saccharomyces cerevisiae*"

7:45–8:15 Stephen Parent, Keith Bostian (Merck Research Labs., Rahway, N.J., USA)
"Protein-DNA and protein-protein interactions regulating the phosphatase multigene family of *Saccharomyces cerevisiae*"

8:15–8:30 (break)

8:30–9:00 Akio Toh-e (Dept. Biology, Univ. Tokyo, Japan)
"Pho85 kinase: a regulator for multiple pathways"

Session III. Transport of Phosphate and Phosphorylated Compounds TUESDAY 14th

8:45–9:00 CONVENERS: Peter Maloney (USA) and Simon Silver (USA)

9:00–9:30 Robert J. Kadner (Dept. Microbiol., Univ. Virginia, USA)
"Transmembrane control of the Uhp sugar-phosphate transport system: the sensation of Glu6P"

9:30–10:00 Peter C. Maloney (Dept. Physiol., Johns Hopkins Univ. Med. Sch., USA)
"Finding the hole in UhpT: applications of molecular biology to a P_i-linked anion exchange carrier"

10:00–10:30 Arthur R. Grossman (Carnegie Inst. of Washington, Stanford, Calif., USA)
"The acclimation of the cyanobacterium *Synechococcus* sp. strain PCC 7942 to nutrient stress"

10:30–10:45 (break)

10:45–11:15 Hiroshi Nikaido (Dept. Molec. & Cell Biol., Univ. California–Berkeley, USA)
"Maltose transport system as an example of ABC-class transporters"

11:15–11:45 Theresa C. Stadtman (NHLBI, Nat. Insts. of Health, USA)
"Selenophosphate: role in selenium donor chemistry"

11:45–12:15 Simon Silver (Dept. Microbiol. & Immunol., Univ. Illinois–Chicago, USA)
Barry P. Rosen (Dept. Biochem., Wayne State Univ., USA)
"Plasmid-determined arsenic resistance"

Lunch, 12:30–2:00 Poster Sessions I–IV, 2:30–4:30 Cocktails, Dinner, 5:30–7:00

Session IV. Phosphate Regulation in Pathogenesis and Secondary Metabolism TUESDAY 14th

7:00–7:15 CONVENERS: John Mekalanos (USA) and Arnold Demain (USA)

7:15–7:45 Alan D. Grossman (Dept. Biology, MIT, USA)
"Signal transduction and development in *Bacillus subtilis*"

7:45–8:15 John Mekalanos (Dept. Microbiol. & Molec. Genetics, Harvard Med. Sch., USA)
"Signal transduction and the control of bacterial virulence"

8:15–8:30 (break)

8:30–9:00 Juan Martín (Dept. Ecol., Genetics, & Microbiol., Univ. León, Spain)
"Phosphate control of antibiotic biosynthesis at the transcriptional level: RNA polymerase, σ factors and phosphate regulated promoters"

9:00–9:30 Samuel I. Miller (Infectious Disease Unit, Massachusetts Gen. Hosp., USA)
"Role of the PhoP regulon in *Salmonella* virulence"

9:30–10:00 Michael L. Vasil (Dept. Microbiol., Univ. Colorado Sch. Med., USA)
"Phosphate and osmoprotectants in the pathogenesis of *Pseudomonas aeruginosa*"

Session V. The Phosphotransferase System WEDNESDAY 15th

8:45–9:00 CONVENERS: Sir Hans Kornberg (UK) and Josef Lengeler (Germany)

9:00–9:30 Gary R. Jacobson (Dept. Biology, Boston Univ., USA)
"Modular structure of the enzymes II of the bacterial phosphotransferase system"

9:30–10:00 Josef W. Lengeler (Dept. Biol./Chem., Univ. Osnabrück, Germany)
"Signal transduction through phosphotransferase systems or PTSs"

10:00–10:30 Pieter W. Postma (E.C. Slater Inst. for Biochem. Res., Univ. Amsterdam, Netherlands)
"The enzymes II of the phosphotransferase system: role in transport and regulation"

10:30–10:45 (break)

10:45–11:15 George T. Robillard (Dept. Biochem., Univ. Groningen, Netherlands)
"The role of domain phosphorylation in the mechanism of mannitol transport via EII^{mtl} of the phosphoenolpyruvate-dependent phosphotransferase system"

11:15–11:30 Saul Roseman (Dept. Biology, Johns Hopkins Univ., USA)
"The bacterial phosphoenolpyruvate : glucose phosphotransferase system"

11:30–11:45 Donald W. Pettigrew (Dept. Biochem. & Biophys., Texas A&M Univ., USA)
"Zn(II)-mediated protein interactions in *E. coli* signal transduction"

11:45–12:15 Milton H. Saier, Jr. (Dept. Biol., Univ. California–San Diego, USA)
"The bacterial phosphotransferase system: a multifaceted regulatory system controlling carbon and energy metabolism"

Lunch, 12:30–2:00

Session VI. Polyphosphates and Phosphate Reserves WEDNESDAY 15th

2:25–2:30 CONVENERS: Arthur Kornberg (USA) and Igor S. Kulaev (Russia)

2:30–3:00 Alan H. Goldstein (Dept. Biology, California State Univ.–Los Angeles, USA)
"Solubilization of exogenous phosphates by gram-negative bacteria"

3:00–3:30 Barry L. Wanner (Dept. Biol. Sci., Purdue Univ., USA)
"P_i-regulated genes for the utilization of phosphonates in *Enterobacteriaceae*"

3:30–3:45 (break)

3:45–4:00 Igor S. Kulaev, remarks

4:00–4:30 Arthur Kornberg (Dept. Biochem., Stanford Univ. Sch. Med., USA)
"Multiple functions of inorganic polyphosphate"

4:30–5:00 Hisao Ohtake (Dept. Fermentation Technol., Hiroshima Univ., Japan)
"Genetic engineering of polyphosphate accumulation in *Escherichia coli*"

Cocktails, 6:00–7:00 Banquet, 7:00–9:00

Session VII. Phospholipids and Protein Export THURSDAY 16th

8:45–9:00 CONVENER: Christian Raetz (USA)

9:00–9:30 Eefjan Breukink (Dept. Biochem. Membranes, Utrecht Univ., Netherlands)
"Lipid involvement in protein translocation in the prokaryotic secretion pathway"

9:30–10:00 William Dowhan (Dept. Biochem. & Molec. Biol., Univ. Texas Med. Sch., USA)
"The role of phospholipids in DNA replication in *Escherichia coli*"

10:00–10:30 Frank Pattus (EMBL, Germany)
"Insertion of pore-forming colicins into membranes: an *in vivo* and *in vitro* study"

10:30–10:45 (break)

10:45–11:15 Christian Raetz (Merck Research Labs., Rahway, N.J., USA)
"Bacterial endotoxins: amazing lipids that activate eucaryotic signal transduction"

11:15–11:45 Gunnar von Heijne (Dept. Molec. Biol., Karolinska Inst., Sweden)
"Membrane protein assembly: can protein-lipid interactions explain the 'positive inside' rule?"

11:45–12:15 William Wickner (Dept. Biochem., Dartmouth Med. Sch., USA)
"Studies of catalytic mechanisms with purified *E. coli* preprotein translocase"

Lunch, 12:30–2:00 Poster Sessions V–IX, 2:30–4:30 Cocktails, Dinner, 5:30–7:00

Session VIII. Protein Export, Structural Studies and Folding THURSDAY 16th

7:00–7:15 CONVENERS: Jan Tommassen (Netherlands) and Jonathan Beckwith (USA)

7:15–7:45 Jonathan Beckwith (Dept. Microbiol. & Molec. Genetics, Harvard Med. Sch., USA)
"Pathways of protein disulfide bond formation *in vivo*"

7:45–8:15 Shoji Mizushima (Tokyo Coll. of Pharmacy, Japan)
"Molecular mechanism of protein translocation across the cytoplasmic membrane of *E. coli*"

8:15–8:30 (break)

8:30–9:00 Linda L. Randall (Dept. Biochem. & Biophys., Washington State Univ., USA)
"Recognition of nonnative structure by the molecular chaperone SecB"

9:00–9:30 Jürg P. Rosenbusch (Dept. Microbiol., Univ. Basel, Switzerland)
"The function of phosphoporin at atomic resolution"

9:30–10:00 Jan Tommassen (Dept. Molec. Cell Biol., Utrecht Univ., Netherlands)
"Biogenesis outer membrane porin PhoE of *Escherichia coli*"

Session IX. Signal Transduction and Phosphoproteins FRIDAY 17th

8:45–9:00 CONVENERS: Ann Stock (USA) and Hideo Shinagawa (Japan)

9:00–9:30 Hideo Shinagawa (Res. Inst. for Microbial Diseases, Osaka Univ., Japan)
"Signal transduction in the phosphate regulon of *Escherichia coli*: dual functions of PhoR as a protein kinase and a protein phosphatase"

9:30–10:00 Shiro Iuchi (Dept. Microbiol. & Molec. Genetics, Harvard Med. Sch., USA)
"Signal transduction in the Arc system for aerobic metabolism in *Escherichia coli*"

10:00–10:30 Austin Newton (Dept. Molec. Biol., Princeton Univ., USA)
"Role of histidine protein kinases and response regulators in bacterial cell division and polar morphogenesis"

10:30–10:45 (break)

10:45–11:15 Alexander J. Ninfa (Dept. Biochem., Wayne State Univ. Sch. Med., USA)
"Regulation of bacterial nitrogen assimilation by the two-component system NRI and NRII (NtrC and NtrB)"

11:15–11:45 Ann Stock (Ctr. for Adv. Biotechnol. & Med., Piscataway, N.J., USA)
"Structure and function of phosphorylated response regulators in bacteria"

11:45–12:15 Andrew Wright (Dept. Molec. Biol., Tufts Univ., USA)
"Modulation of activity of a regulatory protein by PTS-mediated phosphorylation and dephosphorylation controls *bgl* operon expression"

Lunch, 12:30–2:00 Departure

SUMMARIES OF LECTURES AND POSTER ABSTRACTS, BY SESSION

Note: The entries in the poster abstract lists for each session give only the symposium participant occurring first on the abstract. Other author-participants are cross-referenced in the List of Participants at the back.

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"*B. subtilis* alkaline phosphatases controlled by sequential action of genetic switches"

Phosphate, phosphorylated metabolites and Pho regulon

N. N. Rao, A. Kar, J. Yashphe, and A. Torriani

Dept. of Biology, Rm. 16-713, MIT, Cambridge, MA 02139

In *Escherichia coli* a number of genes and operons are involved in the uptake and the assimilation of phosphate (P_i) and phosphorylated compounds. The genes of the Pho regulon induced during P_i-limited growth are regulated by a two-component regulatory system consisting of PhoB and PhoR. An intact Pst system is required for the repression of the Pho regulon under high-phosphate conditions. Therefore, it is likely that the Pst system transduces a signal across the inner membrane which is the primary signal for phosphate limitation. It may be plausible that this primary signal needs to be converted into a secondary cytoplasmic signal. Evidences will be presented to illustrate that P_i triggers the repression of the regulon from outside the cytoplasmic membrane of *E. coli* cells. The intracellular concentration of P_i remains high (3–9 mM) and relatively unchanged during depression of the regulon. Appearance of adenylated nucleotide(s) at the onset of P_i-limitation indicates that they may act as alarmones—a set of signal molecules which alert the cell to the onset of a metabolic stress. A specific nucleotide (dUTP) is shown to be involved in the escape synthesis of alkaline phosphatase during the growth of *E. coli* in excess phosphate.

Mechanism of Transcription Activation of the Pho Regulon by Activator Protein PhoB in *Escherichia coli*

K. Makino*, M. Amemura, S-K. Kim, A. Nakata, and H. Shinagawa
Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan Tel 81-6-875-1606/ Fax 81-6-875-4327

A number of genes in *E. coli*, such as *phoA* and *phoE* genes, are induced by phosphate starvation and constitute a single phosphate (*pho*) regulon. The pair of PhoB and PhoR proteins, which are transcriptional activator and modulator proteins of the *pho* regulon, respectively, is a member of the two-component regulatory systems. PhoR protein undergoes autophosphorylation and catalyzes phosphorylation of PhoB protein. Phosphorylation of PhoB enhances its binding activity to the *pho* box, which is shared by the regulatory region of the *pho* genes, and activates transcription from the *pho* promoters in concert with RNA polymerase holoenzyme containing the major sigma factor, σ^{70}.

The PhoB protein is composed of 229 amino acids. Analysis of numerous PhoB mutants revealed that PhoB contains at least three functional domains; (I) phosphate-accepting domain from phospho-PhoR, (II) DNA(the *pho* box)-binding domain, and (III) a domain interacting with RNA polymerase holoenzyme. To further identify the subunit of RNA polymerase involved in the specific interaction with PhoB, we attempted to isolate *rpo* mutants which are specifically defective in the expression of the *pho* genes. We isolated two *rpoD* mutants with such properties. The *rpoD* mutations altered amino acids within and near the first helix of the putative helix-turn-helix motif in the carboxy-terminal region of σ^{70}. Activities of the *pho* promoters in vivo were severely reduced in these mutants while those of the PhoB-independent promoters were affected only marginally at most. The reconstituted mutant RNA polymerase holoenzymes were severely defective in transcribing the *pstS* gene, one of the *pho* genes, while they were efficient in transcribing the PhoB-independent promoters. Phosphorylated PhoB mediated the specific binding of the wild-type holoenzyme to the *pstS* promoter, while it did not mediate the binding of the mutant holoenzyme variants. These results suggest that PhoB promotes specific interaction between RNA polymerase and the *pho* promoters for the transcription activation, and the first helix of the putative helix-turn-helix motif of σ^{70} plays an essential role in the interaction, probably by directly making contact with PhoB.

Multiple Controls of the *Escherichia coli* PHO Regulon by the P_i Sensor PhoR, the Catabolite Regulatory Sensor CreC, and Acetyl Phosphate

BARRY L. WANNER, *Dept. Biol. Sci., Purdue University, W. Lafayette, IN 47907*

ABSTRACT

Thirty-one (or more) genes are co-regulated as members of the *E. coli* PHO regulon. These genes are arranged in eight transcriptional units, and their gene products probably all have a role in the assimilation of different phosphorus (P) sources from the environment. Their transcription requires an upstream activation site (called a PHO Box) and the phosphorylated form of PhoB as a transcriptional activator. The PHO regulon is subject to multiple controls, and these controls probably all act by affecting phosphorylation of PhoB or dephosphorylation of phospho-PhoB. Its induction by inorganic phosphate (P_i) limitation is a form of transmembrane signal transduction. P_i control involves detection of the extracellular P_i level and is coupled to the first step in P metabolism, P_i uptake. It requires the P_i-specific transporter (Pst), a protein called PhoU, and the P_i sensor PhoR, which can phosphorylate PhoB and which may also dephosphorylate phospho-PhoB. In the absence of PhoR, two P_i -independent controls are evident. These controls are forms of cross regulation. They appear to involve regulatory couplings between P, carbon, and energy metabolism. Both may be coupled to subsequent steps in P metabolism, the entry of P_i into ATP, the primary phosphoryl donor in metabolism. One P_i-independent control requires the catabolite regulatory sensor CreC, which (like PhoR) can phosphorylate PhoB. The other P_i-independent control requires acetyl phosphate, an intermediate in the phosphotransacetylase-acetate kinase (Pta-AckA) pathway for P_i incorporation into ATP. Acetyl phosphate may directly activate the PHO regulon by phosphorylation of PhoB. The first evidence that acetyl phosphate may have a role as an effector of gene regulation resulted from genetic studies on these Pi-independent controls of the PHO regulon. This and other recent work will be discussed. Recent studies from my laboratory concerning these controls of the PHO regulon are described in the citations below.

CITATIONS

Wanner, B. L. and M. R. Wilmes-Riesenberg. 1992. Involvement of phosphotransacetylase, acetate kinase, and acetyl phosphate synthesis in the control of the phosphate regulon in *Escherichia coli*. J. Bacteriol. *174*:2124-2130.

Wanner, B. L. 1992. Minireview. Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria? J. Bacteriol. *174*:2053-2058.

Wanner, B. L. 1993. Gene regulation by phosphate in enteric bacteria. J. Cell. Biochem. *51*:47-54.

Steed, P. M. and B. L. Wanner. 1993. Use of the *rep* technique for allele replacement to construct *pstSCAB-phoU* operon mutants: evidence for a dual role for the PhoU protein in the PHO regulon. (submitted).

THE MOLECULAR MECHANISM OF PHOSPHATE MOVEMENT THROUGH THE PST SYSTEM, AN ABC TRANSPORTER

G.B. Cox and D.C. Webb
John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia

The phosphate specific transport system of *E. coli* is a typical periplasmic permease with a phosphate-binding protein (PstS) located in the periplasm, two integral membrane proteins (PstA and PstC) and a peripheral membrane protein (PstB) on the cytoplasmic side carrying a nucleotide-binding site. The crystal structure of the PstS protein is known and the anhydrous phosphate is bound in a cleft by twelve hydrogen bonds (Luecke & Quiocho, 1990). Two of these hydrogen bonds are from an arginine residue (135) which is salt-bridged to glutamate residues. A pair of proline residues in the PstC subunit, when mutated to alanine, cause the phosphate "channel" to be permanently open. It is proposed that phosphate is translocated through the membrane via a relay of three arginine/glutamate (or aspartate) salt bridges. The necessary movement of the participating helices may be achieved by cis-trans isomerisations of two pairs of proline residues, energised by ATP hydrolysis. Luecke, H. and Quiocho, F.A. *Nature* **347**, 402-406 (1990).

The *E. coli* Ugp transport system is regulated in its activity by internal phosphate

Winfried Boos
Univ. Konstanz, Postfach 5560, 78434 Konstanz, Germany

sn-Glycerol-3-phosphate (G3P) or glyceryl phosphoryl phosphodiesters, the substrates of the *phoB*-dependent Ugp transport system, when transported exclusively through this system, can serve as a sole source of phosphate but not as a sole source of carbon even though the kinetic parameter of the system indicate a high enough flow to support growth (H. Schweizer, M. Argast, and W. Boos, *J. Bacteriol.* **150**: 1154–1163). Using an *ugp-lacZ* fusion we found that the expression of *ugp* does not decline upon exposure to G3P, in contrast to the repressing effect of transport of P_i via the Pst system. This indicated that the Ugp system becomes inhibited after the uptake and metabolism of G3P. Using ^{32}P-labeled G3P we observed that little inorganic phosphate (P_i) is released by cells taking up G3P via the Ugp system but large amounts of P_i are released when the cells are taking up G3P via the GlpT system. Using a *glpD* mutant that could not oxidize G3P but which could still phosphorylate exogenous glycerol to G3P after GlpF-mediated transport of glycerol, we could not find trans inhibition of Ugp-mediated uptake of exogenous ^{14}C-G3P. However, when allowing uptake of P_i via Pst, we observed a time-dependent inhibition of ^{14}C-G3P taken up by the Ugp transport system. Inhibition was completed within 3 min and could be elicited by P_i concentrations below 0.5 mM. Cells had to be starved for P_i in order to observe this inhibition. We conclude that the activity of the Ugp transport system is controlled by the level of internal phosphate.

B. subtilis Alkaline Phosphatases Controlled by Sequential Action of Genetic Switches

F. Marion Hulett, Lei Shi, Wei Liu, Goufu Sun and Ruth Chesnut
Laboratory for Molecular Biology, Department of Biological Sciences
University of Illinois at Chicago

Adaptation by bacteria to environmental signals is often achieved by phosphorylation-dependent signal transduction switches. These switches are composed of two proteins, a sensor kinase and a response regulator. PhoP is homologous to other response regulators and PhoR is homologous to other sensor histidine kinases, and together they comprise a signal transduction switch responsive to environmental phosphate concentrations. When phosphate sources available to *B. subtilis* become limiting, transcription of the *phoPR* operon is induced. Both components of this switch are required for transcription of the *B. subtilis* alkaline phosphatase genes, *phoA* and *phoB*, whose protein products account for greater than 95% of the APase activity induced during phosphate depletion. The kinetics of induction of transcription of the *phoPR* operon and of the APase genes are similar. In a wild type strain, when phosphate becomes limiting and no additional phosphate is supplied to the culture, induction is sustained for approximately 2 hours, after which transcription ceases. A second genetic switch, involving Spo0A, is responsible for stopping induction of the *phoPR* operon and therefore, presumably, transcription of the APase genes, *phoA* and *phoB*. Strains containing mutations in *spo0A*, or in genes of the phosphorelay required for phosphorylation of Spo0A, initiate transcription of *phoPR* and of the APase genes as in the wildtype strains. However, the initial reaction rate is sustained for longer than in the wildtype strain, which results in increased levels of PhoP and in hyperinduction of APases. The role of Spo0A~P is believed to be indirect since Spo0A did not bind to promoters of the *phoPR* operon, or of the APase genes in gel retardation or footprinting studies. *abrB* mutations, in a wildtype background or in a *spo0A* mutant background, have little effect on either transcription of APases or the *phoPR* operon.

Current evidence indicates that when *B. subtilis* experiences phosphate depletion, APases, which are dependent on PhoP and PhoR for expression, are synthesized. Failing to overcome the phosphate deficiency after several hours, the cell abandons the phosphate response and APase accumulation ceases. At that time, the phosphate sensing genetic switch, the *phoPR* operon, is repressed by a second genetic switch, Spo0A~P, which also releases other adaptive response systems from repression by AbrB or enables the cell to sporulate.

POSTER SESSION I (Tuesday Afternoon)

M. Amemura
A new regulatory gene upstream of the *phoM* operon in *Escherichia coli* is a member of the *araC* family

J. Bourne
The effect of phosphate starvation on marine cyanobacterium *Synechococcus* sp. WH7803

J. Combie
Alkaline phosphatase from Yellowstone thermal waters

P. Dunlap
Characterization of *cpdP*, a periplasmic 3′:5′-cyclic nucleotide

A. Kar
Nucleotides as effectors of *phòA* expression in *E. coli*

M. Kasahara
Molecular analysis of the *phoP-phoQ* regulon of *Escherichia coli*

J. Kato
Phosphate taxis and its regulation in *Pseudomonas aeruginosa*

D. N. Ostrovsky
Biosynthesis of a new cyclopyrophosphate is induced in a range of bacteria by redoxcycling chemicals

M. F. Roberts
Cyclic 2, 3-diphosphoglycerate as a component of a new branch in gluconeogenesis in *Methanobacterium thermoautotrophicum* strain ΔH

B. Spira
The relation between ppGpp (magic spot) and the Pho regulon in *Escherichia coli*

G. Sun
Sequential genetic switches in the control of *Bacillus subtilis* alkaline phosphatases

B. Whitton
Surface phosphatase activities in cyanobacteria

J. Yashphe
Effect of *glpT* and *glpD* mutations on expression of the *phoA* gene

A New Regulatory Gene Upstream of the *phoM* Operon in *Escherichia coli* is a Member of the *araC* Family

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Transcription of the genes belonging to the phosphate regulon in *Escherichia coli* is activated by PhoB protein, which is phosphorylated by PhoR protein under phosphate limiting conditions. In the absence of the PhoR function, the genes in the phosphate regulon are expressed constitutively and the expression is dependent on the functions of *phoM* and *phoB*. PhoM is a protein kinase and phosphorylates PhoB. The DNA sequence of the 2.7 kb *Sal* I fragment containing the upstream region of the *phoM* operon was determined. Three open reading frames (*orf*) were found. The *orf-1* lies nearest upstream of the *phoM* operon and encodes a polypeptide with a predicted molecular mass of 33 kDa. The *orf-1* is transcribed in the direction opposite to that of the *phoM* operon. The predicted *orf-1* gene product contains a helix-turn-helix motif and shows significant amino acid similarity to the SoxS protein, a member of the AraC family of transcriptional activators. The transcription initiation site of *orf-1* was determined by primer extension experiment. The ORF-1 protein was purified from an overproducing strain. It was shown by gel retardation assay that the purified ORF-1 protein specifically binds to the 235 bp *Sau*3AI DNA fragment, which contains the upstream regulatory region of *phoM* operon and *orf-1*. The binding site of ORF-1 protein to the fragment was determined by DNase I footprinting, hydroxyl radical footprinting and methylation protection assay. ORF-1 protein protected the DNA segment spanning -11 to -75 from the mRNA start point of *orf-1*. In vitro transcription analysis using RNA polymerase indicated that transcription initiation from the *orf-1* promoter was greatly repressed in the presence of ORF-1 protein. Therefore ORF-1 protein may act as a repressor for own promoter in vivo.

We are examining biologial characteristics of this gene by disruption of *orf-1*, especially whether ORF-1 protein is involved in the control of *phoM* operon or not.

THE EFFECT OF PHOSPHATE STARVATION ON MARINE CYANOBACTERIUM *SYNECHOCOCCUS* SP WH7803

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One of the possible limiting factors for growth of an important group of oceanic primary producers, the pico-planktonic cyanobacteria, is the concentration of inorganic phosphate (Pi). In order to develop a diagnostic method of measuring the extent of phosphate limitation in the natural environment, we set out to determine the effects of phosphate starvation on a laboratory strain, *Synechococcus* SP. WH7803.

One of the more abundant polypeptides found to be synthesised under Pi-limiting conditions, was a 32 kDa protein that was localised to the cell wall. The gene encoding this protein was isolated and characterised. It was found to be a polypeptide of 326 amino acids, that showed 35% identity to PstS, the inducible periplasmic phosphate-binding protein from *E. coli* Immediately downstream of the *pstS* gene is an ORF of 224 amino acids (designated PtrA) which has a helix turn helix DNA binding motif, and shows 30% identity to a group of cyanobacterial transcriptional regulators which include, NtcA, CysR and BifA, themselves members of the Crp/Fnr transcriptional regulator family.

In *E. coli* the high Pi affinity transport system is under the regulation of a two-component sensory system consisting of proteins, PhoR (histidine kinase sensor) and PhoB (response regulator). We have identified two genes from strain WH7803, one of which shows 41% identity to the *phoB* gene of *B. subtilis*, and the other shows 26% identity to the *phoR* gene of *E.coli*. Thus it is possible that the Pi acquisition system in *Synechococcus* sp. WH7803 is under dual regulatory control.

ALKALINE PHOSPHATASE FROM YELLOWSTONE THERMAL WATERS

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Microorganisms inhabiting extreme environments often produce enzymes with subtle differences in the amino acid sequences resulting in markedly more stable enzymes. Geothermal activity creating high temperature environments, serve as a rich source of thermophilic microorganisms. Yellowstone's 10,000 thermal features make up the largest array of geothermal phenomena on earth. This was the source for organisms in this study.

Out of 400 isolates screened, 44 produced substantial levels of alkaline phosphatase. There are numerous cases of enzyme stability at temperatures well in excess of those withstood by the living microorganism producing the enzyme. That proved to be the case for some alkaline phosphatase producing bacteria isolated in this study. Sixteen isolates from habitats between 59 and 76 °C, produced alkaline phosphatase that retained over 50% of its activity after boiling for 1 hour. But this stability was by no means universal. For many, activity was barely detectable following this treatment.

Enzymes produced by thermophiles are generally more active as temperatures increase, although activity can be detected at lower temperatures. For all 10 bacteria producing the most stable alkaline phosphatase, enzyme was readily detected at 20 °C. Activity increased an average of 7.3-fold between 20 and 65 °C.

An enzyme, as the label for an immunological assay is the most unstable component of the system. For example, a reasonable shelf life for alkaline phosphatase requires storage at 4 °C or less. At 45 °C, commercially available calf intestine alkaline phosphatase has a half life of only one month. In home health care test kits, diagnostic kits for doctors offices and fieldable monitors of ecotoxicity and pollution, greater stability under a wide range of conditions is required. This heat stable alkaline phosphatase may meet those requirements.

Characterization of *cpdP*, a Periplasmic 3':5'-Cyclic Nucleotide Phosphodiesterase Gene from *Vibrio fischeri*

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We recently described the novel ability of *Vibrio fischeri*, a common marine bacterium that forms a bioluminescent symbiosis with certain marine animals, to utilize 3':5'-cyclic AMP (cAMP) as a sole source of carbon, nitrogen and phosphorus for growth. This ability was found to correlate with the activity of a 3':5'-cyclic nucleotide phosphodiesterase (3':5'-CNP) of exceptionally high specific activity (8600 μmol AMP produced·min^{-1}·mg protein^{-1}) located in the periplasm of *V. fischeri*, a novel cellular location for this enzyme in bacteria. To examine the relationship between growth on 3':5'-cyclic nucleotides and the periplasmic 3':5'-CNP, and to begin gaining insight into other possible physiological functions of the enzyme, we cloned the gene (designated *cpdP*) encoding it from *V. fischeri* MJ-1. The *cpdP* gene is the first bacterial 3':5'-CNP gene to be cloned. Sequencing and analysis of the 1.26-kilobase (kb) *cpdP* locus revealed a single open reading frame specifying a protein of 330 amino acid residues, including a 22 amino acid leader peptide. The putative *cpdP* promoter contained a reasonable -10 region (TATTAT) but contained no obvious -35 region; instead a 12-base pair inverted repeat (TTAAATATTTAA) occurred just upstream of this location. A possible rho-independent transcriptional terminator with a calculated free energy of -21.2 kcal·mol^{-1} followed the CpdP protein coding sequence. The predicted subunit MW of 33,636 for the mature CpdP protein (36,087 Da less 2451 Da for the leader peptide) was consistent with the MW of 34,000 estimated by SDS-PAGE. The deduced amino acid sequence of the CpdP protein exhibited 30.3% identity with the low affinity 3':5'-CNP (PDE1) of *Saccharomyces cerevisiae* and 33.6% identity with the extracellular 3':5'-CNP of *Dictyostelium discoideum.* A gene replacement mutant of *V. fischeri* MJ-1 containing a 0.45-kb *Bgl*II deletion within the *cpdP* gene lacked periplasmic 3':5'-CNP activity and did not grow on cAMP, which confirmed in *V. fischeri* the relationship between *cpdP*, synthesis of the periplasmic 3':5'-CNP, and growth on cAMP. The mutant exhibited no obvious sensitivity to high concentrations of cAMP (5 mM and 10 mM), which indicates the enzyme probably does not play a role in defense against extracellular cAMP. The periplasmic location and high specific activity of the CpdP protein suggest it functions in scavenging the carbon, nitrogen and phosphorus from 3':5'-cyclic nucleotides in the environment or possibly in some aspect of animal-bacterial interaction in bioluminescent symbiosis.

Nucleotides as effectors of *phoA* expression in *E. coli*

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The repression of the synthesis of alkaline phosphatase (AP) is implemented by six genes: *phoR*, which codes for the sensor—a transmembrane protein kinase—and the five genes of the P_i transport operon Pst (*pstS, pstC, pstA, pstB, phoU*). A mutation in *phoR* provokes constitutive synthesis of AP by cross-talks with other kinases not P_i-regulated.

A mutation in any one of the *pst* genes also provokes a constitutive synthesis of AP. Analysis of the nucelotide pool of *E. coli* isolated by acid extraction of ^{32}P-labeled cells and fractionated by HPLC showed that some nucleotides were accumulated at P_i starvation. We previously presented results showing that two of these nucleotides (S_2 and S_{15}) were required for synthesis of AP in plasmolysed cells. Here we present further evidence and analysis of the effect of S_2. This nuceotide which is detected in *pst* mutants comigrates with dUTP on PEI thin-layer chromatography. dUTP itself was less effective as compared to S_2 in the *in vitro* synthesis of alkaline phosphatase.

By immunoblot it is evident that dUTP provokes a *de novo* synthesis of AP *in vitro*. A mutant *dut-1* (BW285 *dut-1*) presented an escape synthesis of AP in conditions of repressibility (excess P_i) due to accumulation of dUTP.

We postulate that the nucleotide S_2 (a possible analogue of dUTP) may serve as a positive signal to the regulatory operon *phoB-phoR* to turn on the Pho regulon.

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Molecular analysis of the *phoP - phoQ* regulon of *Escherichia coli*

The *phoP - phoQ* operon belongs to a large family of two - component regulatory systems, which modulate expression of sets of genes responding to environmental changes. The features of *E. coli phoP - phoQ* operon are very similar to those of *S. typhimurium*, which are involved in the regulation of the expression of the Salmonella virulence genes and the *phoN* gene which codes for a nonspecific acid phosphatase. Although *E. coli* K-12 has no virulent phenotype and lacks the *phoN* gene, it still has the *phoP - phoQ* operon. To elucidate the physiological roles of *E. coli phoP - phoQ* regulon, we attempted to identify the proteins and the genes regulated by the *phoP- phoQ* system. We constructed strains lacking the *phoP - phoQ* or *phoQ* genes. Experiments employing transcriptional fusions show that the *phoP - phoQ* operon is induced by glucose, phosphate and nitrogen starvation, and that it is positively autoregulated. Cellular protein patterns as analyzed by computer - aided two - dimensional gel electrophoresis showed that synthesis of more than 80 protein species were affected by the *phoP - phoQ* or *phoQ* deletions, indicating the importance of the *phoP- phoQ* system for the cell physiology. Several proteins, whose synthesis was greatly influenced, were analyzed by a micro - sequencing method. We found that the *malE* gene product (maltose binding protein) was much increased in the *phoQ* deletion mutant and the *udp* gene product (uridine phosphorylase) was much decreased in the *phoP- phoQ* deletion mutant.

Phosphate taxis and its regulation in *Pseudomonas aeruginosa*

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Phosphorus compounds are essential constituents in organisms. To deal with phosphate limitation, bacteria have evolved complex regulatory systems to assimilate phosphorus very efficiently. Motile bacteria are known to behaviorally respond to a wide range of chemical stimuli, including amino acids, sugars, aromatics and organic acids. Though many works on bacterial chemotaxis have been reported, no paper seems to report about the chemotactic response to phosphate in bacteria. In this presentation, we demonstrate phosphate taxis and its regulation in *Pseudomonas aeruginosa* PAO1.

The chemotactic response toward phosphate was determined by a rapid capillary assay technique which is a modification of Adler's procedure. Only phosphate-starved cells were attracted to phosphate, indicating the phosphate taxis is induced by phosphate limitation. Some mutants, constitutive for alkaline phosphatase, showed the chemotactic response to phosphate, regardless of whether the cells were starved for phosphate. The products of *phoB* and *phoR* genes are known to regulate the expression of a number of genes, including the structural gene for alkaline phosphatase (*phoA*), which are inducible by phosphate limitation. To investigate whether the phosphate taxis in *P. aeruginosa* is also regulated by the *phoB* and *phoR* gene products, a *phoB*$^-$ mutant was isolated by the gene replacement technique. The *phoB*$^-$ mutant strain still exhibited the inducible phosphate taxis, indicating that the phosphate taxis is not regulated by the *phoB* and *phoR* products.

Curiously, the mutants, constitutive for alkaline phosphatase as well as phosphate taxis, were found to be more resistant to arsenate than was the strain PAO1. This seems to suggest that a relationship may exist between the regulation of phosphate taxis and phosphate transport in this organism. A 23-kb *Hin*dIII fragment, which is able to restore the inducible phosphate taxis of the constitutive mutants, was cloned from the chromosomal DNA of strain PAO1 using a broad-host-range cosmid vector. After being sucloned, the nucleotide sequence of the 7.4-kb *Bam*HI fragment is now being determined.

Biosynthesis of a new cyclopyrophosphate is induced in a range of bacteria by redoxcycling chemicals .

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Nitrofuran drugs,dipyridyle pesticides and some other chemicals were shown to induce biosynthesis of a new macroergic compound 2-methylbutane-1,2,3,4-tetraol-2,4-cyclopyrophosphate in Brevibacteria,Rhodococci,Mycobacteria and related species.The phosphorus of this compound may constitute about 50% of the cell NMR-visible phosphorus,its accumulation being independant of de novo protein synthesis and correlates with the ability of the producin bacteria to survive in a medium supplemented with the drugs.

Ostrovsky et al.,BioFactors (1992) v.3,261-264 ;(1992) v.4,63-68
FEBS Lett.(1992) v.298,159-161

"Cyclic 2,3-diphosphoglycerate as a component of a new branch in gluconeogenesis in *Methanobacterium thermoautotrophicum* strain ΔH"

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A unique compound, cyclic 2,3-diphosphoglycerate (cDPG) is the major soluble carbon and phosphorus solute in *M. thermoautotrophicum* ΔH under optimal conditions of cell growth. It is a component of an unusual branch in gluconeogenesis in these bacteria. $^{13}C_2$-acetate-pulse/^{12}C-acetate-chase and ^{31}P NMR methodology was used to observe the relationship between other phosphorylated metabolites of this branch. In these experiments *M. thermoautotrophicum* cells were induced *in vivo* to accumulate large amounts of 2-PG and 2,3-DPG by incubation at 50°C for prolonged periods of time. Upon a return to the growth temperature the cells can be induced to grow exponentially under conditions where 2-PG and 2,3-DPG, rather than cDPG are the major solutes. While the total concentration of these three phosphorylated molecules (cDPG + 2,3-DPG + 2-PG) is maintained, rapid interconversion of ^{13}C label between these three phosphorylated species was observed. An examination of the degree of ^{13}C specific labeling showed that in exponentially growing cells ^{13}C content per mol of phosphorylated species was highest in 2-PG and decreased in the order 2-PG → 2,3-DPG → cDPG → a large molecule(s) not visible in the high resolution NMR spectrum. The reverse reactions could predominate in stationary phase. Implication of a polymeric compound which can exchange label with cDPG in this path was provided by ^{13}C NMR studies (1-D and 2-D INADEQUATE) of solubilized cell debris. Possibly this biosynthetic pathway connects the phosphorylated three carbon intermediates pool of gluconeogenesis with some precursors of amino acid synthesis. In that case when methanogenesis can supply acetyl-CoA in abundance, a large intracellular cDPG pool would help to balance carbohydrate and protein synthesis. On the contrary, under starvation conditions it would facilitate the balance of macromolecule degradation with ATP synthesis.

The Relation Between ppGpp (Magic Spot) and the Pho Regulon in *Escherichia coli*

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Wild type *E. coli* cells starved for P_i induce the synthesis of guanosine-3′, 5′-bispyrophosphate (ppGpp), also known as "magic spot." The synthesis of ppGpp is known to be induced under other conditions of stress as starvation for amino acids (the stringent response) or for a carbon source. ppGpp synthesis can be catalyzed by either of two pathways. One pathway, activated during starvation for amino acids, is via the ribosomal bound enzyme ppGpp synthetase I (PSI), coded by the gene *relA*. The alternative pathway, via the *spoT*-encoded enzyme PSII, is activated during carbon starvation.

ppGpp synthesis during P_i-starvation is obeserved in a *relA*-deletion mutant but not in a *relA-spoT* double deletion mutant and therefore it is catalyzed by PSII. Alkaline phosphatase is derepressed in the *relA-spoT* mutant and therefore ppGpp is not an effector in the regulation of alkaline phosphatase (and probably the entire Pho regulon). Likewise, overproduction of ppGpp from a plasmid does not induce the synthesis of alkaline phosphatase in the presence of P_i. In a *pho*-constitutive strain (*pstS* mutant) ppGpp synthesis is still induced by starvation for P_i. However, in a *phoB*-negative mutant its synthesis is strongly inhibited though no "pho box" is evident in the promoter of *spoT*. Thus, the P_i-dependent synthesis of ppGpp is somehow related to one or more of the Pho regulon genes.

Sequential Genetic Switches in the Control of Bacillus subtilis Alkaline Phosphatases

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Current evidence indicates that when *B.subtilis* experiences phosphate depletion, alkaline phosphatase(s)(APases), which are dependent on *phoPR* for expression,are synthesized. Failing to overcome the phosphate deficiency after several hours, the cell abandons the phosphate response and the APase accumulation ceases. At that time, the phosphate-sensing genetic switch, the *phoPR* operon, is repressed by a second genetic switch, Spo0A~P.

Several lines of evidence suggest that APase expression is directly regulated by the *phoPR* operon which encodes members of a two-component system composed of PhoP, the response regulator and PhoR, the sensor kinase. Preliminary gel retardation studies show that the PhoP protein, in crude cell extracts of *B.subtilis* or from the lysate of an *E.coli* strain overexpressing PhoP, binds to the promoter region of *phoA*.

The *phoPR* operon is both positively and negatively regulated. PhoP serves as a positive transcription regulator of the *phoPR* operon. An internal deletion within the *phoP* gene resulted in low-level, constitutive expression of *phoP* comparable to 30% of induced, wild- type transcription levels. The expression of the *phoP* gene is also negatively regulated by Spo0A~P, a response regulator of another two-component system. Mutations in *spo0A* or in genes of the phosphorelay required for the phosphorylation of Spo0A, *spo0F* and *spo0B*, enhance *phoP* expression three fold. APase production in the *spo0A* background also increases five to six fold. The kinetics of the induction of transcription of the *phoPR* operon and of the APase genes are similar in the *spo0A* mutant.

The expression of APase genes is also positively regulated by a third two-component system, ORFX17 and ORFX18. Current experiments are directed at understanding whether ORFX17/18 act directly at the promoter regions of the APase genes or through the regulation of *phoPR* or *spo0A*.

SURFACE PHOSPHATASE ACTIVITIES IN CYANOBACTERIA

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All 50 axenic strains of cyanobacteria tested showed 'inducible' surface phosphomonoesterase activity (PMEase), 47 also showed phosphodiesterase (PDEase) and 35 showed phytase activities. Phosphomonoesterase activity was demonstrated by measurement of p-NPP and 4-methylumbelliferyl phosphate hydrolysis and the ability of the organism to grow using a range of organic phosphate substrates.

Many of the strains showing the highest activity per unit biomass belong to the Rivulariaceae (*Calothrix*, *Rivularia*, *Gloeotrichia*), which are characterized by the development of tapered ends to the trichomes under conditions of moderate P-limitation; in some cases the tapered parts of the trichome form long colourless multicellular hairs. During batch culture of two strains of *Calothrix* forming hairs (D253 = PCC 7709; D550), detectable PMEase activity and hair formation commenced at about the same stage; this corresponds to when the mean cellular P content fell to 0.7% dry weight. Histochemical staining shows that all of the surface PMEase activity of *Calothrix* D253 and much of that of *Calothrix* D550 is associated with the hair; however, extracellular PMEase (but not PDEase) activity is also present.

Studies are underway to isolate the *pme* gene in *Calothrix* D253. A genomic library has been produced and several different approaches have been / are being applied to obtain the gene(s). Heterologous probing indicates that a putative gene has little similarity to the *phoA* of *Synechococcus* PCC 7942 or the gene encoding a tyrosine/serine phosphatase in *Nostoc* UTEX 584, which is apparently responsible for surface phosphatase activity in that strain.

Studies are also in progress to characterize the features of the environments where Rivulariaceae dominate in nature. All sites investigated show marked differences in ambient P concentration with time, with occasional pulses of much higher levels of phosphate, much of which if often organic. The organisms exist in a P-limited state for much of the time, but reproduce (form hormogonia) after exposure to a sufficiently long pulse of P and then become N-limited for a short period, before again becoming P-limited.

Effect of *glpT* and *glpD* Mutations on Expression of the *phoA* Gene in *Escherichia coli*

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The function of the *phoA* promoter (measured by β-galactosidase activity in a *phoA-lacZ* fusion strain) was repressed when *glpT*$^+$ cells, which possessed the sn-glycerol-3-phosphate (sn-G3P) transport system geared for its utilization as a carbon source, were utilizing sn-G3P as a sole source of phosphate. However, this promoter was fully active in a *glpT* defective mutant grown at the same conditions (The cells were devoid of alkaline phosphatase activity). Since *glpT* is the only route for the exit of orthophosphate (Pi) from the cell at the experimental conditions, it was concluded that the repression of the activity of the *phoA* promoter was due to the Pi excreted into the periplasm or to the medium, rather than due to a variation in the level of cytoplasmic Pi. This conclusion was supported by *in vivo* ^{31}P nuclear magnetic resonance analysis of *E. coli* cells which showed that the intracellular concentration of Pi remained constant in the wild type as well as in the *glpT* mutant whether the cells were grown on excess (2mM) Pi or on sn-G3P as a phosphate source. Blocking of the utilization of sn-G3P through the glycolytic pathway by a *glpD* mutation did not prevent the repression of the *phoA* promoter, nor did it affect the derepression observed in the *glpT* mutant. These findings suggest the lack of a negative regulator molecule in the glycolytic pathway, and the possibility that a metabolite in the glycerophospholipid biosynthetic pathway may act as an activator.

2:30–4:30 **Round Table. Structure-Function of Alkaline Phosphatase**

Evan Kantrowitz, Moderator (Dept. Chemistry, Boston College, USA)

Cathy Brennan (Abbott Labs., Abbott Park, Ill., USA)

Joseph E. Coleman (Dept. Molec. Biophys. & Biochem., Yale Univ., USA)

Eunice Kim (Vertex Pharmaceuticals, Cambridge, Mass., USA)

Albert Matlin (Dept. Chemistry, Oberlin College, USA)

Harold W. Wyckoff (Dept. Molec. Biophys. & Biochem., Yale Univ., USA)

Session II. Regulation of Phosphate Metabolism in Yeast MONDAY 13th

7:00–7:15 CONVENERS: Yasuji Oshima (Japan) and Harlyn Halvorson (USA)

7:15–7:45 Yasuji Oshima (Dept. Biotechnol., Osaka Univ., Japan)
"The regulatory circuit for the phosphatase genes in *Saccharomyces cerevisiae*"

7:45–8:15 Stephen Parent, Keith Bostian (Merck Research Labs., Rahway, N.J., USA)
"Protein-DNA and protein-protein interactions regulating the phosphatase multigene family of *Saccharomyces cerevisiae*"

8:15–8:30 (break)

8:30–9:00 Akio Toh-e (Dept. Biology, Univ. Tokyo, Japan)
"Pho85 kinase: a regulator for multiple pathways"

The regulatory circuit for the phosphatase genes in Saccharomyces cerevisiae

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Transcription of the PHO5 gene, encoding repressible acid phosphatase, and the PHO84 gene, encoding a P_i transporter, in S. cerevisiae is repressed by P_i in the medium. The P_i signals are conveyed to these genes through a system composed of at least five gene products, from the PHO2 (= BAS2, GRF10), PHO4, PHO80, PHO81, and PHO85 genes. A current model proposes that, in low-P_i medium, a positive regulatory protein, Pho4p encoded by PHO4, activates transcription of PHO5 and PHO84 in combination with Pho2p. In high-P_i medium, a negative regulatory protein, Pho80p, interacts directly with Pho4p and prevents transcription of PHO5 and PHO84. When the P_i concentration in the medium is sufficiently low, Pho81p inhibits the function of Pho80p, or eliminates the P_i (or a derivative of it) that activates Pho80p, thus allowing Pho4p to transcribe the PHO structural genes. Transcription of PHO81 is also under the regulation of Pho4p, indicating that the regulatory system forms a closed circuit. This yeast also has repressible alkaline phosphatase encoded by PHO8, mostly in the vacuole. The same regulatory system, except for PHO2, functions for regulation of PHO8 transcription. The PHO85 gene encodes an analog of protein kinases and is thought to act as an activator of Pho80p.

We have examined cis-acting regulatory regions of the PHO5, PHO8, PHO81, and PHO84 genes. We found that a copy of 5'-CACGTG-3' sequence is effective in the PHO8 promoter for binding and function of Pho4p. The 5'-CACGTT-3' and 5'-CACGTG-3' sequences were found, respectively, in the UASp1 and UASp2 boxes of the PHO5 promoter. The same 5-'CACGTG-3' sequence is located in the PHO81 promoter at nucleotide position -344 from the putative ATG codon of this gene and is in the center of a 20-bp sequence protected by Pho4p from DNase I digestion in vitro. Similar examination of the PHO84 promoter, we found that it bears five copies of 5'-CACGT(G/T)-3' motif at nucleotide positions -880 (site A), -587 (B), -436 (C), -414 (D), and -262 (E). Deletion analyses and base substitutions in the 6-bp motifs revealed that proximal two, one is either C or D and the other is E, are essential and enough for full regulation of PHO84 transcription. Comparison of these Pho4p binding sites in the PHO5, PHO8, PHO81, and PHO84 promoters and the CDE I element, 5'-RTCACRTG-3'(R = purine), suggested that a G residue flanked on the 5' end of the 6-bp motif is also important for the specific binding and for function of Pho4p.

Protein-DNA and Protein-Protein Interactions Regulating the Phosphatase Multigene Family of *Saccharomyces cerevisiae*. S. Parent,[1] M. Justice,[1] L.-W. Yuan,[2] J. Hopper,[2] K. Bostian,[1,3] 1) Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065 2) Penn. State Univ. College of Medicine, Hershey, PA 17033 3) Microcide Pharmaceuticals, 4040 Campbell Ave., Menlo Park, CA 94025

In *Saccharomyces cerevisiae* the expression of acid and alkaline phosphatases involved in controlling phosphate metabolism is regulated transcriptionally in response to external Pi concentrations by a complex, genetically-defined system. Transcription of the genes encoding the repressible acid phosphatases (*PHO5*, *PHO10*, and *PHO11*) is regulated by both positive (*PHO2*, *PHO4*, and *PHO81*) and negative (*PHO80* and *PHO85*) regulatory proteins. Pho4 and Pho2 belong to conserved basic-helix-loop-helix and homeodomain families of sequence-specific DNA-binding proteins, respectively. We previously reported that overexpression of *PHO4* suppresses the nonderepressible phenotype of *pho2* mutants and confers constitutive expression of acid phosphatase in wild type cells. To understand the molecular basis of these phenotypes, we examined the regulatory interactions of Pho2, Pho4 and Pho80.

Using yeast cell extracts and antibodies recognizing Pho4 and Pho2, we examined interactions of these proteins with *PHO5* UAS elements in gel-shift assays. Sequences required for Pho4 binding to UASp1 (CACGTT) and UASp2 (CACGTG) are important for phosphate-regulated expression of UASp-*CYC1*-LacZ reporter genes. Several observations suggest that these UAS elements are regulated differentially. Although, the affinity of Pho4 for UASp2 was greater than for UASp1, point mutations which reduced Pho4 binding to either element lowered *PHO5* promoter activity to comparable levels. A promoter containing a UASp1 up-mutation (CACGTG) which increased Pho4 *in vitro* binding to UASp1 was more active than the wild type promoter. Moreover, a promoter containing the UASp1 up-mutation (CACGTG) and a mutation which inactivates UASp2 (CACGCG) expressed wild type levels of *PHO5*. We also demonstrated that Pho2 binds several regions of the *PHO5* promoter, including UASp1. Mutations that decrease Pho2 binding at UASp1 inactivated this element. These results suggest that cooperative interactions between Pho4 and Pho2 are important for UASp1 regulation and that Pho2 plays multiple roles in controlling *PHO5* expression.

Under repressing conditions, Pho4-mediated transcriptional activation is inhibited by Pho80 and Pho85 proteins. Physiological and genetic data suggest that Pho4 and Pho80 interact physically, but direct evidence is lacking. We employed the two-hybrid system to demonstrate that Pho4 and Pho80 interact to form a stable complex. Moreover, in this system, this interaction occurs in cells grown under repressing and derepressing conditions. These results suggest that repressing and derepressing states are not regulated by controlling the ability of Pho80 to interact with and inhibit Pho4.

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The PHO85 gene has been isolated as one of negative regulators of the phosphate control system of Saccharomyces cerevisiae. The amino acid sequence of Pho85 protein deduced from its nucleotide sequence indicates that it belongs to the Cdc28 family kinases; amino acid alignment of Cdc28 and Pho85 shows 51% identical amino acids. Moreover, Pho85 protein contains almost complete PSTAIR sequence which is a hallmark of Cdc28 family proteins. Inspite of such a high homology between Cdc28 and Pho85, these protein exert their distinct function. To explore functions of the conserved domains of Pho85 kinase, we introduced a point mutation(s) in these regions by oligonucleotide-directed mutagenesis and tested the effect of each mutation on cell growth and PHO regulation.

Phenotypes of the PHO85 disruptant The fact that the pho85 disruptant can germinate and grow on YPD, although slower than wild type strain, indicates that the PHO85 gene is dispensable. The pho85 disruptant display pleiotropic effects such as Gal^-, Suc^-, or Pet^-. All these traits can be suppressed simultaneously by a $pho4^-$ mutation.

Characterization of PHO85Y18F The 18th tyrosine (Y) residue, which corresponds to the 15th Y of $cdc2^+$ of S. pombe, has been substituted with phenylalanine (F), resulting in the $Pho85^-$ phenotype when the mutant gene is on a low copy number plasmid. This is consistent with our observation that Pho85Y18F protein has a lower kinase activity. This result is in clear contrast to the facts that the 15th Y exerts an important regulatory role in Cdc2 kinase and that Y15F substitution activates H1 kinase activity. Unexpectedly, overproduction of PHO85Y18F protein by the GAP-DH promoter or PHO85Y18F on a multicopy plasmid made production of repressible acid phosphatase constitutive.

Point mutations in the PSTAIR region To understand the function of the PSTAIR region further, alanine scanning was conducted in this region. Every mutant gene tested, expressing from its own promoter or from the GAP-DH promoter, was introduced into the pho85 strain or wild type strain. Every mutant gene so far examined on a low copy vector is more or less pho85 defective. However, mutants except pho85E53A and pho85K58A showed the $Pho85^+$ phenotype when they overexpressed from a strong promoter or on a multicopy vector. These exceptional mutants were constitutive even when they expressed from their own promoter on a low copy number vector.

Pho85 kinase The GST-PHO85 fusion gene was constructed to facilitate assaying Pho85 kinase. We confirmed that this fusion gene has PHO85 activity. Among proteins tested (histon H1, myelin basic protein, protamine, and k-casein), only k-casein can be used as substrate. Pho85 kinase activity stayed at a constant level during phosphate deprivation, indicating that it is unlikely that Pho85 kinase activity is directly regulated by phosphate concentration. We also found that the $pho81^{ko}$ or $pho80^{ko}$ strain possesses a normal level of Pho85 kinase activity.

G. Dhinakar

The effect of monoses on the subcellular distribution of phosphatases in yeast

THE EFFECT OF MONOSES ON THE SUBCELLULAR DISTRIBUTION OF PHOSPHOTASES IN YEAST

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Yeast cells have been widely known to bring about fermentation process through the operation of anaerobic breakdown of monoses. The uptake of sugars from the ambient medium largely depends on the cellmembrane. Subcellular distribution of acid and alkaline phosphotases has been analysed through ultracentrification techniques.

The activity levels of acid and alkaline phosphotases in different cell fractions such as cellmembrane, nuclei, lysozomes, mito chondria, microsomes and cytosols. Interesting trends have been noticed in the subcellulas distribution of phosphotases, which have been correlated with the intracellular transport of phosphorylated sugars for the metabolic activities of the cell.

The pattern of distribution of phosphotases was dependent on the type of monose present in the medium. The monoses such as glucose, fructose and galactose have shown different patterns of distribution of phosphotases in the subcellulas fractions. Thus variability in the phosphotase activities depending on the monoses in the medium has been envisaged.

Similarly the activitiy levels of phosphotases in different subcellular fractions were also found to be modulated by the concentration of the monose in the ambient medium. Thus the concentration dependent activity pattern of phosphotase in the yeast cells has been visualised.

The fermentation activity seems to be dependent on the type and concentration of monoses in the ambient medium. Since the activity of phosphotases will be obligatory for the process of fermentation, nutrient mediated activity pattern of the phosphotases in the yeast cells has been proposed.

Session III. Transport of Phosphate and Phosphorylated Compounds TUESDAY 14th

8:45–9:00 CONVENERS: Peter Maloney (USA) and Simon Silver (USA)

9:00–9:30 Robert J. Kadner (Dept. Microbiol., Univ. Virginia, USA)
"Transmembrane control of the Uhp sugar-phosphate transport system: the sensation of Glu6P"

9:30–10:00 Peter C. Maloney (Dept. Physiol., Johns Hopkins Univ. Med. Sch., USA)
"Finding the hole in UhpT: applications of molecular biology to a P_i-linked anion exchange carrier"

10:00–10:30 Arthur R. Grossman (Carnegie Inst. of Washington, Stanford, Calif., USA)
"The acclimation of the cyanobacterium *Synechococcus* sp. strain PCC 7942 to nutrient stress"

10:30–10:45 (break)

10:45–11:15 Hiroshi Nikaido (Dept. Molec. & Cell Biol., Univ. California–Berkeley, USA)
"Maltose transport system as an example of ABC-class transporters"

11:15–11:45 Theresa C. Stadtman (NHLBI, Nat. Insts. of Health, USA)
"Selenophosphate: role in selenium donor chemistry"

11:45–12:15 Simon Silver (Dept. Microbiol. & Immunol., Univ. Illinois–Chicago, USA)
Barry P. Rosen (Dept. Biochem., Wayne State Univ., USA)
"Plasmid-determined arsenic resistance"

Transmembrane Control of the Uhp Sugar-Phosphate Transport System: The Sensation of Glu6P.

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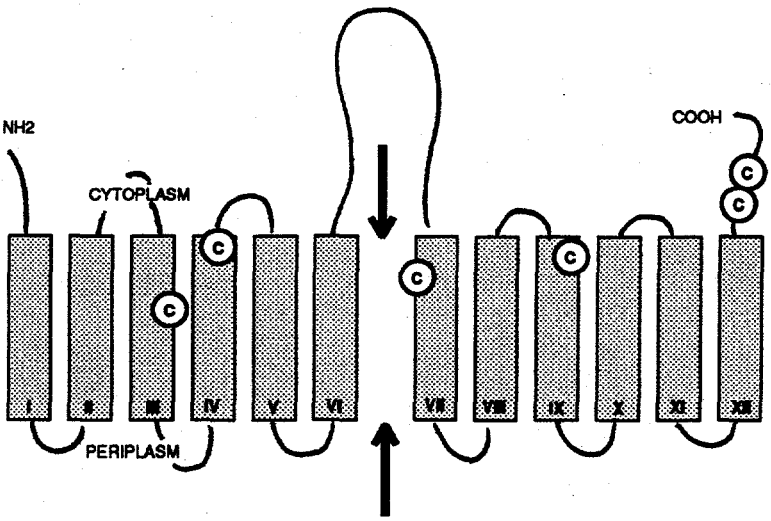
The *uhp* locus of *Escherichia coli* encodes a transport system that mediates the accumulation in unaltered form of a number of organo-phosphate compounds. The UhpT transport protein catalyzes the electroneutral exchange of organo-phosphates, such as glucose-6-phosphate (Glu6P), with inorganic phosphate. It bears amino acid sequence homology to other organophosphate-exchange proteins, such as the glycerol-3-phosphate transporter, GlpT, and the phosphoglycerate transporter, PgtP. The induction of the UhpT transport system by external Glu6P is dependent on the three products of the *uhpABC* gene cluster, located immediately upstream of *uhpT*, and is subject to catabolite repression. The UhpA and UhpB proteins possess sequence similarities to the response-regulator and sensor-kinase proteins of two-component bacterial regulatory systems, respectively, whereas UhpC is related to the UhpT family of transport proteins. The membrane-associated UhpB and UhpC proteins appear to function as a complex in signaling. Randomly sited mutations in either *uhpB* or *uhpC* can confer constitutive Uhp expression but the activity of most of the constitutive mutants in UhpB was dependent on the presence of an active form of UhpC, even though UhpB otherwise lies downstream of UhpC in the signaling pathway.

The UhpA protein is absolutely required for Uhp transcription and is predicted to comprise several modules: a phosphorylation module similar in sequence to CheY, a nonconserved linker region, and an activation module conserved among many transcription activator proteins and predicted to contain a helix-turn-helix motif. Deletions that truncated UhpA from its carboxyl end were prepared and showed that removal of even 8 amino acids caused lost of transcription activation activity. Deletions ending within the activation module conferred a dominant-negative phenotype against the wild-type allele, and the site of this interference is examined. The catabolite activator protein, CAP, activates *uhpT* transcription by 10 to 20-fold and mediates the catabolite repressibility of Uhp expression. The DNA site for CAP binding in the *uhpT* promoter is centered at -103.5, relative to the start of transcription, and exhibits face-of-the-helix periodicity when it is moved away from the rest of the promoter. The protein surface region on the CAP protein that is involved in activation at many CAP-dependent promoters is not necessary for activation at the *uhpT* promoter, suggesting the operation of a different mode of CAP action from that at other CAP-dependent promoters. The catabolite repressibility of Uhp expression may be to prevent excessive flux of metabolites through the glycolytic pathway, a condition which can lead to several types of growth inhibition or killing.

Finding the hole in UhpT: Applications of molecular biology to a Pi-linked anion exchange carrier. Run-Tao Yan & Peter C. Maloney. Department of Physiology, Johns Hopkins University Medical School, Baltimore, MD 21205. (Tel: 410-955-8325; Fax: 410-955-0461)

Escherichia coli uses a Pi-linked anion exchange system to transport glucose 6-phosphate (G6P). This protein, known as UhpT, is thought to resemble other secondary carriers in having a hydrophobic core with 12 transmembrane segments (see figure); this model of secondary structure that has been largely confirmed by Kadner and his colleagues with the help of gene fusion technology. We have taken a closer look at UhpT and have tried to find the path taken by substrate as it moves through the protein. The work has relied mainly on directed mutagenesis, with a particular focus on an analysis of cysteines and the use of impermeant sulfhydral agents. UhpT has six cysteine residues, at positions 108, 143, 265, 331, 436 and 436 (see circles), and a preliminary study showed that none of these is required for function. This let us concentrate our attention on C143 and C265, the residues imparting sensitivity to *p*-chloromercuribenzosulfonate (PCMBS), an impermeant sulfhydral agent resembling G6P in size, shape and charge. We showed that C143 was exposed to the cytoplasm, as expected from hydropathy analysis, but we found no sidedness for C265. Rather, *C265 was accessible to PCMBS from both membrane surfaces* (see arrows). Since the attack at C265 was blocked by G6P, we conclude that position 265 lines the substrate translocation pathway. By implanting new cysteines and then testing their reactivity to PCMBS, we hope to learn a great deal more about this domain in UhpT.



A Low Resolution View of UhpT

The Acclimation of the Cyanobacterium *Synechococcus* sp. Strain PCC 7942 to Nutrient Stress. Arthur R. Grossman, Devaki Bhaya and Jackie L. Collier. The Carnegie Institution of Washington, Department of Plant Biology, 290 Panama Street, Stanford, CA 94305.

We are studying the affect of nutrient limitation on the acquisition of the limiting nutrient and changes in the internal structure of the cyanobacterium *Synechococcus* sp. Strain PCC 7942. We have concentrated most of our work on the sulfur stress responses. When starved for sulfur *Synechococcus* sp. Strain PCC 7942, and other cyanobacteria, increase their capacity to take up sulfate and other sulfur-containing compounds. The genes encoding components of some of the systems involved in the transport of sulfur-containing compounds have been sequenced and important regions of the promoters of these genes are being defined. The internal structure of the cell also dramatically changes when the cells are starved for sulfur (and nitrogen). Photosynthetic oxygen evolution declines to nearly zero, the photosynthetic membranes are attenuated and there is a rapid and ordered degradation of the light harvesting phycobilisome. We have recently isolated mutants that are unable to degrade the phycobilisome during nutrient-limited growth. Complementation of these mutants has led to the isolation of a gene (*nbl*) encoding a small polypeptide that is required for phycobilisome degradation. The transcript for this gene accumulates to high levels when the cells are starved for either sulfur or nitrogen. Furthermore, when this gene is expressed on a multicopy plasmid, the phycobilisome is degraded even when the cells are maintained in nutrient replete medium. Another gene (*txl*), whose product resembles thioredoxin, as well as a disulfide isomerase, also may be regulated when the cells are nutrient limited. The *txl* gene product appears to regulate the functioning and/or assembly of photosystem II. Details of the specific and general nutrient stress responses of cyanobacteria will be discussed.

Maltose Transport System as an Example of ABC-class Transporters

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"ABC (ATP-binding casette)" transporters are composed of multiple subunits or domains, and are widely distributed in prokaryotes and eukaryotes. Periplasmic binding protein is usually one of the components of the prokaryotic members of this group, including the phosphate transport system of *E. coli*, and a major question is why such binding proteins are needed for transport.

The maltose transporter system of *E. coli* is composed of a soluble, periplasmic maltose-binding protein (MBP, the MalE product), and a membrane-associated transporter composed of four subunits, one each of MalF and MalG, and two copies of the ATPase subunit, MalK. The system carries out an uphill transport of maltose and maltodextrins by utilizing the energy of ATP hydrolysis.

In the wild-type strain, the binding of the ligands to MBP is essential for transport. Starting from a mutant with a nonpolar deletion in *malE*, Treptow and Shuman (1985) isolated several mutants that can now transport maltose in the absence of MBP. These mutants are altered in the putative transmembrane subunits, MalF and MalG. When the membrane-associated transporter from these mutants was reconstituted into lipid vesicles, we found that the mutant transporter catalyzed rapid hydrolysis of ATP. In contrast, the wild-type transporter did not hydrolyze ATP, until both MBP and the ligand, maltose, were added. It thus appears that a major function of the liganded MBP is to act as a transmembrane signaling molecule, and to inform the MalK ATPase, located on the other side of the membrane, of the presence of the ligands to be transported.

The role of MBP, however, may not be limited to this. The MBP-independent mutant transporters fail to transport maltodextrins in intact cells (Treptow and Shuman, 1985). Although this could be due to their difficulty in crossing the outer membrane, our recent data suggest that they are not transported by membrane vesicles of these mutants. Possibly MBP is needed for maltodextrins, but not for maltose, because the former have to be "guided" into the transport channel by MBP. Interestingly, Gehring et al. (1991) showed that MBP binds maltodextrins in two ways, "end-on" or "sideways". When the reducing end of the maltodextrins are altered by reduction or oxidation, they cannot be transported although they bind tightly to MBP (Ferenci et al., 1986). Our recent studies showed that these modified maltodextrins bind to MBP exclusively in the sideways manner. Thus it may be that maltodextrins must bind in the end-on manner to MBP, in order to enter the transporter channel in a correct manner.

Selenophosphate: role in selenium donor chemistry

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A reactive selenium donor compound required for the biosynthesis of certain selenium-dependent enzymes and seleno-tRNAs has been identified as monoselenophosphate ($HSePO_3H_2$). This compound is formed from ATP and selenide by selenophosphate synthetase, the bacterial selD gene product, as shown in reaction 1.

ATP + selenide → selenophosphate + orthophosphate + AMP (1)

The highly oxygen-labile enzyme product was purified and shown to be indistinguishable from chemically synthesized monoselenophosphate by ^{31}P NMR spectroscopy and ion pairing HPLC. The two compounds can be used interchangeably as substrate for the enzymic conversion of 2-thiouridine residues in tRNAs to 2-selenouridine residues, a reaction in which selenium is substituted for sulfur. The mechanism of biosynthesis of selenophosphate from ATP and selenide was studied using mutant forms of selenophosphate synthetase prepared by site specific mutagenesis. A cysteine residue (Cys-17) and a lysine residue (Lys 20) in a glycine rich region near the amino terminus were shown to be essential for catalytic activity.

Plasmid-determined arsenic resistance. Barry P. Rosen and Simon Silver, Wayne State University, Detroit, Michigan; and University of Illinois, Chicago, Illinois

The bacterial resistance to arsenate, arsenite and antimony is governed by many plasmids, both in Gram positive and Gram negative bacteria. It is of interest to researchers of phosphate metabolism, since (a) the oxyanion arsenate is an analog for phosphate in transport systems and enzymes, (b) the arsenic efflux system is an ATPase, mechanistically quite different from that of the Pst phosphate uptake system, and (c) reduction of arsenate to arsenite (and oxidation of arsenite to arsenate) occurs with bacterial resistance systems. The arsenate, arsenite, and antimony resistance system of E. coli plasmid R773 consists of five genes and determines a membrane efflux ATPase; the comparable system of S. aureus plasmid pI258 consists of three genes and appears to function as a chemiosmotic efflux pump. The ArsB membrane proteins are 58% identical at the amino acid level between the E. coli and S. aureus efflux systems. Chimeric proteins that are 1/3 from one source and 2/3rds from the other are functional as well. The membrane protein from E. coli plasmid R773 couples with a soluble (ArsA) ATPase subunit to function as a membrane ATPase. Dimeric ArsA sits on ArsB, and each ArsA subunit contains two (related) ATP binding sites, for a total of four per complex. Both sites are needed, and can be provided in a single polypeptide or by complementation of separate half-proteins (from half genes) synthesized from different plasmids. The arsA gene is missing from two independently sequenced versions of staphylococcal Ars (which appears to function in response to the membrane potential and to be independent of ATP). R773 ArsB is non-functional in the presence of mutant ArsA protein but functions as a chemiosmotic carrier (as well) in the total absence of ArsA. The third component of the arsenic resistance determinant is ArsC, the intracellular arsenate reductase enzyme. Arsenate is reduced to arsenite which is then rapidly effluxed from the cells, providing resistance. ArsC is a small monomeric protein that functions with redox energy from reduced thiols, from thioredoxin in vitro with the pI258 version of ArsC, but the source of electrons for the R773 version of ArsC (they share only 18% identical amino acids) is uncertain.

POSTER SESSION III (Tuesday Afternoon)

B. Persson
Construction and expression of a biotinylated phosphate permease fusion protein

CONSTRUCTION AND EXPRESSION OF A BIOTINYLATED PHOSPHATE PERMEASE FUSION PROTEIN.

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The derepressible phosphate permease encoded by the *PHO84* gene in *Saccharomyces cerevisiae*, is a hydrophobic, polytopic plasma membrane protein of 596 amino acids that catalyzes the coupled transport of phosphate with protons.
Genes encoding novel fusion proteins have been constructed by recombinant DNA methodologies. These constructs allows for the expression of chimeric polypeptides; consisting of the amino terminal 264 or 596 residues of the phosphate permease from *S. cerevisiae* and a 100 residue domain derived from the oxalacetate decarboxylase α subunit gene of *Klebsiella pneumoniae*. The fusion proteins are subject to *in vivo* biotinylation, when expressed in *Escherichia coli*. This property is conferred by the presence of the decarboxylase domain which is recognized by the endogenous biotin ligase. Attachment of the *in vivo* biotinylatable peptide to the PHO84 carboxyl-terminus allows one to exploit the biotin binding protein, avidin, which has an extremely high affinity for this cofactor. Covalent modification of the fusion proteins with biotin resulted in the expression of permease fusion proteins which may be purified with the use of a monomeric avidin column. An engineered factor Xa protease cleavage site at the fusion junction can be used to remove the biotin peptide sequence if desired. This technique represents a highly efficient method for purification of the PHO84 permease which may be applicable for other membrane proteins in general.

Session IV. Phosphate Regulation in Pathogenesis and Secondary Metabolism TUESDAY 14th

7:00–7:15 CONVENERS: John Mekalanos (USA) and Arnold Demain (USA)

7:15–7:45 Alan D. Grossman (Dept. Biology, MIT, USA)
"Signal transduction and development in *Bacillus subtilis*"

7:45–8:15 John Mekalanos (Dept. Microbiol. & Molec. Genetics, Harvard Med. Sch., USA)
"Signal transduction and the control of bacterial virulence"

8:15–8:30 (break)

8:30–9:00 Juan Martín (Dept. Ecol., Genetics, & Microbiol., Univ. León, Spain)
"Phosphate control of antibiotic biosynthesis at the transcriptional level: RNA polymerase, σ factors and phosphate regulated promoters"

9:00–9:30 Samuel I. Miller (Infectious Disease Unit, Massachusetts Gen. Hosp., USA)
"Role of the PhoP regulon in *Salmonella* virulence"

9:30–10:00 Michael L. Vasil (Dept. Microbiol., Univ. Colorado Sch. Med., USA)
"Phosphate and osmoprotectants in the pathogenesis of *Pseudomonas aeruginosa*"

Signal transduction and development in *Bacillus subtilis*

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Mechanisms by which cells begin to differentiate into specialized cell types is intimately related to the detection and transduction of both internal and external signals and the alteration of gene expression in response to those stimuli. Our work focuses on the control of differentiation, gene expression, and signal transduction in the bacterium *Bacillus subtilis.* Under appropriate conditions, cells of *B. subtilis* can enter a developmental pathway that leads to the formation of dormant heat resistant endospores. Signals required for initiation of the sporulation pathway include nutrient deprivation, high cell density, continued DNA synthesis, undamaged DNA, and an intact citric acid cycle. Only when all of these conditions are met will the cells efficiently enter into the sporulation pathway. We have shown that all of these conditions control the activation (phosphorylation) of the developmental transcription factor encoded by *spo0A*. Spo0A is a response regulator of the family of two component regulatory systems. It is a DNA binding protein that can activate and repress transcription, based on the location of its binding site. The activity of Spo0A is controlled by phosphorylation. Unlike other response regulators, phosphorylation of Spo0A is regulated by a multicomponent phosphorelay. Cells sense and integrate the multiple developmental signals by controlling the phosphorylation pathway. We suggest that each condition needed for the initiation of sporulation affects one or more steps in the phosphorylation pathway. In this way, enough Spo0A~P accumulates only when all conditions favor sporulation.

Spore formation is not the only developmental pathway available to *B. subtilis*. Under other conditions, a small fraction of the cells in a culture develop the ability to be transformed with exogenous DNA. This state of genetic competence occurs when cultures reach high cell density in a minimal glucose medium. The cell density signals that induce the development of competence seem to control the phosphorylation of a response regulator, the *comA* gene product. ComA~P binds to DNA and activates transcription of additional regulatory genes needed for competence development.

The cell density signals are mediated by a pheromone that accumulates in culture medium as cells grow to high density. We have purified and characterized a cell density factor that induces the development of genetic competence. The factor is a modified peptide containing 9 or 10 amino acids and seems to be processed from a precursor of approximately 60 amino acids. The cellular response to the competence factor requires the oligopeptide permease encoded by *spo0K*. Characterization of Spo0K, an ABC transporter, should help determine of the competence factor needs to be transported back into the cell, or if it sends a transmembrane signal to activate the competence pathway.

SIGNAL TRANSDUCTION AND THE CONTROL OF BACTERIAL VIRULENCE

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The expression of known virulence factors is regulated by environmental condidtions in vitro that presumably reflect similar cues present in host tissues. The identification of many virulence factors has been both dependent on, and limited by, our ability to mimic these host environmental signals in the laboratory. Under laboratory condidtions a variety of different signals have been shown to modulate virulence gene expression. These include the temperature of incubation, media parameters (i.e., osmolarity, salinity, pH, organic substrates, trace metalic ions), and gases (oxygen and carbon dioxide). In many cases this modulation of virulence gene expression has been traced through genetic studies to regulatory genes with traditional motifs (e.g., two component regulatory systems, AraC homologs, etc.). In some cases, these regulatory proteins have had an unusual structure that does not fit into known motifs easily. For example, ToxR, the virulence regulator of Vibrio cholerae, is a membrane protein that is homologous to the C-terminal portion of OmpR but lacks all other sequences that would suggest that phosphorylation plays any role in its activity. Fusion protein analysis instead suggests that dimerization may be important to ToxR activity and by analogy to other two component regulatory proteins as well.

Although we have made progress in identifying the regulatory genes and signals controlling virulence gene expression in the laboratory, we are still trying to make sense out of the sometimes paradoxical nature of these regulatory responses. The true metabolic and physiological state of bacteria during infection remains matter for conjecture.

In order to circumvent some of these problems, we have devised a genetic system, termed IVET (in vivo expression technology), that positively selects for bacterial genes specifically induced during the infection cycle. Such in vivo induced (ivi) genes are poorly expressed on laboratory media but undergo dramatically elevated expression in host tissues. Two different types of IVET vectors have been studied. The first begins with a bacterial strain carring a mutation in a biosynthetic gene that renders it attenuated in a given animal model. Growth of the mutant strain is then complemented by operon fusions to the same biosynthetic gene. The second method involves making operon fusions to tnpR, a gene encoding a site-specific recombinase. In the latter case, induction of the ivi::tnpR gene fusion is monitored in animal tissues by the appearance of site-specific recombinants that are easily scored by virtual of their antibiotic resistance phenotype. These IVET systems have been studied in two pathogenic bacterial species, Salmonella typhimurium and Vibrio cholerae.

We have shown that mutations in ivi genes confer virulence defects, thus showing that this class of genes plays an important role in pathogenesis. The nature of ivi gene products will provide a more complete description of the metabolic, physiological, and genetic factors that contribute to the virulence of bacterial pathogens.

PHOSPHATE CONTROL OF ANTIBIOTIC BIOSYNTHESIS AT THE TRANSCRIPTIONAL LEVEL: RNA POLYMERASE, SIGMA FACTORS AND PHOSPHATE REGULATED PROMOTERS.

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Phosphate controls negatively the biosynthesis of a variety of antibiotics (Martin and Demain, 1980; Martin, 1989). To elucidate the molecular mechanisms of phosphate control we have used as model system the biosynthesis of candicidin, which is strongly regulated by phosphate concentrations above 1 mM (Liras et al, 1977).

p-Aminobenzoic acid (PABA), the precursor of the p-amino-acetophenone moiety of candicidin is synthesized from chorismic acid by PABA synthase. The synthasis of PABA synthase is repressed by concentrations of Pi in the range of 1-10 mM. The pabS gene of S. griseus, encoding PABA synthase was cloned (Gil et al 1985); it forms part of a cluster of genes related to candicidin biosynthesis (Criado et al 1992). The pabS transcript reached a peak at 12 h of incubation in batch cultures, preceeding the formation of PABA synthase and the antibiotic itself. Inorganic phosphate (7.5 mM) reduced the synthesis of the pabS transcript by 90-95% and consequently the formation of PABA synthase and candicidin. However phosphate stimulated two to three fold total RNA synthesis. The 1.7 kb pabS transcript shown by Northern hybridization was greatly reduced in amount in cells grown in 7.5 mM phosphate. Phosphate-deregulated mutants were impaired in the transcriptional control exerted by phosphate.

In order to characterize the transcriptional mechanisms involved in expression of the pabS gene, the core RNA polymerase of S. griseus has been purified to near homogeneity. To obtain large amount of sigma factors, three genes encoding sigma factor related proteins have been cloned from S. griseus. One of them, hrdB has been shown to be involved in primary metabolism. Transcription of the hrdB gene is stimulated in phosphate rich medium but it is switched off after nutritional shift-down conditions which trigger antibiotic biosynthesis and sporulation in solid medium. A second sigma factor gene, hrdD, has also been cloned and sequenced. Disruption of this gene is in progress to establish if the sigma factor encoded by hrdD is required for expression of antibiotic biosynthetic genes. It seems likely that the transcription of genes for antibiotic biosynthesis and sporulation is achieved by specific sigma factors which will recognize a particular class of promoters. We propose that such promoters are also recognized by DNA-binding proteins which modulate gene expression in response to phosphate. DNA binding proteins which interact with two different phosphate-regulated promoters of S. griseus have been shown by gel retardation.

Role of the PhoP Regulon in *Salmonella* Virulence.

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The PhoP regulon, comprised of the genes regulated by the PhoQ (sensor-kinase) and PhoP (transcriptional-regulator) proteins, is necessary for *Salmonella typhimurium* mouse virulence, survival within macrophages, and growth on succinate as a sole carbon source. *phoP* and *phoQ* mutants have increased sensitivity to pH 3.0 and cationic antimicrobial proteins (defensins and magainins) derived from diverse sources such as mammalian neutrophils, intestinal crypt epithelium (Paneth cells), and reptile skin.

Loci termed *phoP* activated genes require PhoP/PhoQ for transcriptional activation. These loci are widely spaced on the chromosome and have been estimated to encode approximately 20 proteins. One locus, *phoN*, encodes a 27kD peptide which as a dimer functions as a nonspecific periplasmic phosphatase with a pH 5.5 optimum. Another well defined locus, *pagC* , encodes an 18 kD outer membrane protein with similarity to a number of proteins of diverse phenotypes, including a*Yersinia enterocolitica* epithelial cell invasion protein (Ail), a bacteriophage lambda outer membrane protein (Lom), a serum resistance protein (Rck) of *Salmonella typhimurium* , and a *Enterobacter cloacae* protein (OmpX) whose expression alters porin transcription and antibiotic sensitivity. Several other loci that require PhoP/PhoQ for expression have been defined as transcriptional and translational gene fusions formed by transposons.

In contrast to the regulation of the nonspecific alkaline phosphatase of *E. coli,* whose synthesis is promoted in response to low phosphate concentration, the regulation of acid phosphatase synthesis and*pag* transcription is enhanced 70-100 fold when bacteria are within acidified macrophage phagosomes. Transcriptional induction requires acidification of the phagosome below pH 5.0, which occurs approximately 4 hours after phagocytosis. Starvation, acid shock to pH 4.5., and entering stationary phase all increase expression of *pag* loci, though 10-20 fold less than that observed when bacteria arewithin macrophage phagosomes. Starvation induced expression is nonspecific and may be induced by limitation of either nitrogen, carbon, sulfur, or phosphate.

A mutation in PhoQ (phenotype PhoP constitutive) that simulates signal transduction and activates *pag* expresion has been defined. This mutation is in the periplasmic and presumed sensor domain of PhoQ. Single amino acid changes in this periplasmic domain can significantly reduce or activate gene expression. Experiments indicate that wild type and activated PhoQ are co-dominant consistent with the possibility that signal transduction is accomplished by intermolecular interaction of PhoQ proteins.

Strains with the PhoQ activating mutation are similar to PhoP/PhoQ deleted bacteria in that they are attenuated for survival within macrophages and mouse virulence. Morphologic evaluation of *Salmonella* interactions with macrophages indicate that the organism enters macrophages by macropinocytosis and resides within spacious phagosomes, membrane bound organelles that are similar to macropinosomes formed by growth factors and transforming agents. PhoP activated gene expression is correlated with the eventual shrinkage and acidification of these spacious phagosomes. PhoP constitutive bacteria are defective in the early phase of survival within macrophages and fail to induce macropinocytosis and spacious phagosome formation. These strains synthesize decreased amounts of a number of proteins encoded by *phoP* repressed genes. The defined *phoP* repressed loci encode envelope proteins; one locus called *prgH* , is essential for *Salmonella typhimurium* mediated endocytosis by epithelial cells. We hypothesize that PhoP/PhoQ controls two phases of *Salmonella* gene expression mediated by contact with eukaryotic cells: early events involve the expression of *phoP* repressed genes and later intracellular macrophage signals modulate the expression of PhoP activated genes which promote intracellular survival of the microorganism.

PHOSPHATE AND OSMOPROTECTANTS IN THE PATHOGENESIS OF *PSEUDOMONAS AERUGINOSA*

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It is now clear that the capacity of a bacterial pathogen to colonize and ultimately produce disease is dependent upon its ability to respond to a variety of stressful environmental conditions that may be encountered in the organs and tissues of a host. These responses will include, the de novo expression of genes that allow the invading organism to survive in this hostile environment, and the induction of toxic products (e.g., toxins) which damage host defenses and tissue. The stressful environmental conditions an organism could encounter in the infectious process will include, limitations of specific nutrients such as iron or phosphate, changes in temperature and increased osmotic pressure.

P. aeruginosa produces two phospholipase C (PLC) enzymes that have been implicated in virulence. Both PLCs hydrolyze phosphatidylcholine, which is the major component of lung surfactant and eucaryotic cell membranes, but not bacterial membranes. One PLC, designated PLC-H, is hemolytic and cytolytic for leukocytes. The second PLC (PLC-N) is non-hemolytic. PLC-H is posttranslationally modified by the products of two overlapping genes that are in the same operon 3' to the gene encoding PLC-H. These gene products, PLCR1 and PLCR2, alter the conformation of PLC-H and increase its hemolytic activity.

Expression of both PLCs is derepressed during phosphate (Pi) limited growth. Both PLC operons are transcriptionally activated by PhoB, a DNA binding protein that activates several Pi regulated genes in *P. aeruginosa* and is homologous to PhoB of *E. coli*. Choline and its derivatives have been reported to induce acid phosphatase expression in *P. aeruginosa* and serve as osmoprotectants for many procaryotic organisms. Low concentrations (1mM) of phosphorylcholine, one of the substrate products of PLC-H and PLC-N, choline and its derivatives, betaine and dimethylglycine, but not monomethylglycine (sarcosine), induce PLC-H expression in Pi sufficient conditions (>10mM). This induction of PLC-H also occurs in a *phoB* deletion mutant that is nonderepressible for PLC production in low Pi. RNase protection assays suggest that there are two transcriptional initiation sites for the operon encoding PLC-H, one for osmoprotectant dependent induction and one for Pi-starvation dependent induction, while there is only one transcriptional initiation site for *plcN* regardless of the presence or absence of osmoprotectant. These results suggest that choline, or some factor activated by the presence of choline is able to substitute for PhoB as a transcriptional activator of the PLC-H operon. These and other results also indicate that expression of PLC-N is dependent upon *phoB* even in the presence of osmoprotectants.

It is clear, that the regulation of just two (PLCs) of the many virulence determinants of this opportunistic bacterial organism involves an eminently complex microbial response to specific relevant environmental conditions.

POSTER SESSION IV (Tuesday Afternoon)

L. Banta

Modulation of *Agrobacterium tumefaciens* VirA activity by carbohydrates: a single residue outside the predicted ChvE binding site is crucial for sugar enhancement of phenolic perception

Modulation of *Agrobacterium tumefaciens* VirA activity by carbohydrates: A single residue outside the predicted ChvE binding site is crucial for sugar enhancement of phenolic perception

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The soil bacterium *Agrobacterium tumefaciens* infects susceptible plants, resulting in unregulated cell division and crown gall tumor formation. During the infection process, a specific DNA molecule (the T-DNA) derived from a bacterial tumor-inducing (Ti) plasmid is integrated into the plant genome. T-DNA processing and transfer are mediated by a number of Ti plasmid-encoded virulence (Vir) proteins. Expression of the *vir* genes is induced upon perception of substituted phenolics such as acetosyringone (AS) exuded by wounded plant cells. Genetic studies have shown that the *virA* and *virG* gene products are required for this transcriptional activation. VirA spans the inner membrane, with two transmembrane domains, a large periplasmic region, and a C-terminal cytoplasmic tail. In analogy to the "sensor" proteins in several other bacterial "two-component regulatory systems" with which it shares sequence similarity, VirA is believed to play a central role in signal perception and transduction. The VirA protein contains an autophosphorylation site within its carboxy terminus and can transfer the phosphate directly to VirG. Like other "activator" proteins in the "two-component regulatory systems", VirG acts as a positive regulatory element, binding to a cis-acting regulatory sequence upstream of the inducible *vir* operons and presumably activating transcription from these promoters.

The complexity and subtlety of the control over *vir* gene induction is underscored by the finding that phenolic sensitivity is significantly enhanced by the presence of certain carbohydrates, many of which are major components of plant cell walls. Mutations in the chromosomally encoded ChvE protein result in a loss of this sugar enhancement and a drastic reduction in virulence. ChvE, which is homologous to two periplasmic proteins involved in sugar recognition and uptake in *Escherichia coli* , is thought to interact with the periplasmic domain of VirA. Deletion of most of this domain abolishes sugar-induced enhancement of *vir* activation. Taken together, these observations are consistent with a model in which the ChvE protein senses the presence of inducing sugars and synergistically influences *vir* induction by altering the ability of VirA to respond to phenolics.

In this study, we demonstrate that mutations within the periplasmic domain can drastically alter the sensitivity of VirA to AS. Using site-directed mutagenesis, we have characterized the role of three individual amino acids in sugar-dependent AS sensitivity, and have correlated the induction phenotype with the tumorigenic capacity of strains expressing the mutant versions of VirA. Substitution of leucine for Glu^{255} abolishes sugar enhancement, while replacement with aspartic acid results in a wild-type phenotype. This residue lies outside the predicted ChvE binding domain and thus identifies a new region of the VirA periplasmic domain crucial for the synergistic enhancement of *vir* gene induction by carbohydrates.

Session V. The Phosphotransferase System WEDNESDAY 15th

8:45–9:00 CONVENERS: Sir Hans Kornberg (UK) and Josef Lengeler (Germany)

9:00–9:30 Gary R. Jacobson (Dept. Biology, Boston Univ., USA)
"Modular structure of the enzymes II of the bacterial phosphotransferase system"

9:30–10:00 Josef W. Lengeler (Dept. Biol./Chem., Univ. Osnabrück, Germany)
"Signal transduction through phosphotransferase systems or PTSs"

10:00–10:30 Pieter W. Postma (E.C. Slater Inst. for Biochem. Res., Univ. Amsterdam, Netherlands)
"The enzymes II of the phosphotransferase system: role in transport and regulation"

10:30–10:45 (break)

10:45–11:15 George T. Robillard (Dept. Biochem., Univ. Groningen, Netherlands)
"The role of domain phosphorylation in the mechanism of mannitol transport via EII^{mtl} of the phosphoenolpyruvate-dependent phosphotransferase system"

11:15–11:30 Saul Roseman (Dept. Biology, Johns Hopkins Univ., USA)
"The bacterial phosphoenolpyruvate : glucose phosphotransferase system"

11:30–11:45 Donald W. Pettigrew (Dept. Biochem. & Biophys., Texas A&M Univ., USA)
"Zn(II)-mediated protein interactions in *E. coli* signal transduction"

11:45–12:15 Milton H. Saier, Jr. (Dept. Biol., Univ. California–San Diego, USA)
"The bacterial phosphotransferase system: a multifaceted regulatory system controlling carbon and energy metabolism"

MODULAR STRUCTURE OF THE ENZYMES II OF THE BACTERIAL PHOSPHOTRANSFERASE SYSTEM. Gary R. Jacobson, Dept. of Biology, Boston Univ., 5 Cummington St., Boston, MA 02215. Tel: 617-353-4708; Fax: 617-353-6340.

The bacterial phosphoenolpyruvate (PEP)-dependent carbohydrate phosphotransferase system (PTS) is responsible for the transport and phosphorylation of a variety of carbohydrates in many bacteria. A phospho (P)-group is first sequentially transferred from PEP to 2 general phosphotransfer proteins of the PTS, Enzyme I and HPr, both of which are phosphorylated on His residues. P-HPr then transfers its P-group to an integral-membrane, carbohydrate-specific Enzyme II (EII) or EII-complex, which in all PTS's consists of at least 3 domains or proteins: IIA, the P-acceptor from P-HPr (on a His residue); IIB, the P-acceptor from P-IIA (on a Cys or His residue) and the P-donor to the substrate; and IIC, the integral-membrane domain responsible for recognition and transport of the substrate. In *E. coli* these domains may be within one polypeptide [mannitol-specific EII (EII-mtl)], comprise 2 proteins (glucose-specific EIICB/EIIA pair), or be 3 separate proteins (the cellobiose-specific PTS). Regardless of the organization, the roles of the domains and the mechanism of phosphorylation and transport of all EII's are likely to be similar. One of the best-studied EII's of the PTS is EII-mtl. The roles of its domains, and the phosphotransfer steps outlined above, have been well-established by a combination of biochemical, biophysical and molecular genetic techniques. Recent studies of this and related PTS EII's have concentrated on the structure and transport mechanism of the IIC domain, and on how phosphorylation of the IIA/B domains influences the activity of IIC. Gene fusion analyses suggest that IIC traverses the membrane 6-8 times, and other studies have shown that it is sufficient to recognize and transport its substrate. However, phosphorylation of IIA and IIB greatly increases the transport activity of IIC. Moreover, recent studies have revealed an important role for a 90-residue segment in IIC, which is at least partially exposed in the cytoplasm of the cell. This region contains a conserved His residue and a highly conserved GIXE motif, both of which are important for substrate-binding, transport and phosphorylation. Mutagenesis and mutant complementation studies suggest that this segment in IIC interacts with the IIB domain in coupling phosphorylation to transport in EII-mtl.

Signal transduction through phosphotransferase systems or PTSs

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The sum of all phosphotransferase systems or PTSs of a bacterial cell form a central regulatory and signal transduction system. This system couples carbohydrate transport, peripheral catabolism as well as the chemotactical behaviour of a cell, and integrates information from the environment obtained through transport systems and from the intracellular energy state as reflected in the phosphorylation level of its proteins. The information is then used to regulate through transcription anti-termination, inducer exclusion, catabolite repression, and chemotaxis the behaviour of the cell.

Two general proteins (Enzyme I or EI, and Histidin protein or HPr) and several substrate specific Enzymes II (EII) form the PTS. Each EII comprises three autonomous domains involved in substrate binding and translocation (IIC or channel) or in substrate phosphorylation (IIA, formerly EIII, and IIB). During transport and signal transduction the four domains EI, HPr, IIA and IIB are phosphorylated (in that sequence) We have analysed their structure through sequence alignment techniques and their function through deletion of domains, homologous and heterologous exchange of such domains, localised mutagenesis of essential amino acid residues and the selection of mutants with specific defects in transport and signal transduction. We will present data describing the sequence of events involved in transport and PTS-dependent chemotaxis. We will discuss the results in relation to other signal transduction systems, in particular two-component systems, and global regulatory networks, and point out several striking analogies.

The Enzymes II of the phosphotransferase system: role in transport and regulation

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The bacterial phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) catalyzes the transport and phosphorylation of numerous carbohydrates in Gram-negative and Gram-positive organisms. The phospho group of PEP is transferred via two general proteins, Enzyme I and HPr, and a number of carbohydrate-specific Enzymes II (EII's) to the respective carbohydrates. All PTS proteins are phospho proteins in which the phospho group is attached to a histidine residue or, in a number of cases, a cysteine residue.

The carbohydrate-specific Enzymes II are unable to transport substrates in the absence of phosphorylation, e.g. in *ptsH,I* mutants lacking HPr and/or Enzyme I. We have studied in enteric bacteria the glucose PTS that consists of the cytoplasmic $EIIA^{Glc}$ (site of histidine phosphorylation) and the membrane-bound $EIICB^{Glc}$ (site of cysteine phosphorylation). Mutations have been isolated in the *Escherichia coli ptsG* gene, encoding $IICB^{Glc}$, which allow transport of glucose without phosphorylation. The mutations have been sequenced and are localized in a segment of 150 amino acid residues that forms a cytoplasmic loop between two membrane-spanning segments of $IICB^{Glc}$. The so-called "uncoupled" EII^{Glc} can still phosphorylate glucose when Enzyme I, HPr and IIA^{Glc} are added. Phosphorylation, but not transport, via these mutant EII's is abolished when the Cys421 residue, which is phosphorylated in wild type II^{Glc}, is replaced by a serine residue.

EII's are important in controlling bacterial metabolism since they determine the phosphorylation state of the central regulatory protein $EIIA^{Glc}$. $EIIA^{Glc}$ is phosphorylated via Enzyme I/HPr and dephosphorylated via $IICB^{Glc}$. Phosphorylated IIA^{Glc} is involved in activating adenylate cyclase and non-phosphorylated IIA^{Glc} inhibits a number of proteins involved in the uptake and metabolism of non-PTS compounds, i.e. the lactose and melibiose permease, MalK, and glycerol kinase. We will discuss mutant IIA^{Glc} proteins that are defective in regulation of transport but that are still able to activate adenylate cyclase. By varying the amount of $IICB^{Glc}$, using a regulatable *ptsG* gene, it can be shown that $IICB^{Glc}$ controls to a large extent the glucose flux, as measured by glucose transport and phosphorylation. Enzyme I, HPr and IIA^{Glc} have no control. We will discuss the implications of these findings for regulation of carbohydrate metabolism in enteric bacteria.

The Role of Domain Phosphorylation in the Mechanism of Mannitol Transport via EII^{mtl} of the Phosphoenolpyruvate-Dependent Phosphotransferase System

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Flow dialysis studies of mannitol binding to right-side-out and inside-out *E. coli* membrane vesicles have demonstrated the existence of three stages of binding in the transport process, binding to a site oriented towards the periplasm, binding to a site oriented towards the cytoplasm and binding to an occluded site which is reachable from the cytoplasmic side but whose access is kinetically restricted. Data from these studies also indicates that facilitated diffusion occurs under certain circumstances, however, the process is very slow. Phosphorylation speeds up the transport process by at least a factor of 1000 presumably by lowering the activation energy for the isomerization of the binding sites, however, what this means in physical terms remains undefined. The purpose of the work to be presented is to gain insight into the details of the phosphoryl transfer step between the phosphorylated B domain and mannitol bound to the membrane-bound C domain.

Since it is accepted that the EII^{mtl} monomer can catalyze PEP-dependent mannitol phosphorylation but that the dimer is necessary to catalyze Mtl/Mtl-P exchange we have started from these two premises and compared the kinetics of IIC^{mtl}-dependent mannitol phosphorylation and exchange, using i. isolated $IIBA^{mtl}$ ii. wild-type EII^{mtl} and iii. a chimeric EII consisting of the EII^{glc} membrane-bound domain (IIC) fused to the EII^{mtl} cytoplasmic domains (IIBA). The chimer enables us to separate the mannitol binding site and the phosphorylation sites by creating heterodimers consisting of one mannitol-binding C domain plus one chimeric protein lacking a mannitol-binding C domain but possessing a phosphorylatable BA^{mtl} domain.

The combined kinetic data lead to a model for the coupled movement of sites linked to the driving force of phosphorylation.

THE BACTERIAL PHOSPHOENOLPYRUVATE:GLYCOSE PHOSPHOTRANSFERASE SYSTEM

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III^{Glc} is a key PTS protein in *E. coli* and *S. typhimurium*. In glucose transport, III^{Glc} is a phosphate carrier in the following P-transfer sequence: PEP → Enzyme I → HPr → III^{Glc} → II^{Glc} → sugar. But III^{Glc}, which is encoded by the *crr* gene, plays essential roles in other phenomena, such as diauxie. It interacts with (and regulates) glycerol kinase, possibly a glycerol permease/kinase complex, the lactose, melibiose and maltose permeases, and adenylate cyclase. Recent collaborative studies between this laboratory and those of Drs. S. J. Remington (X-ray crystal structures), D. Torchia (NMR), E. Freire (microcalorimetry), and D. W. Pettigrew (glycerol kinase) have provided a wealth of structural information on III^{Glc} and the crystal structure of a III^{Glc}/glycerol kinase complex.

III^{Glc} from *E. coli* (18,100 kDa) is very stable structurally. Following heat denaturation, it regains both its phosphoryl transfer activity and its enthalpy of denaturation. It contains 2 domains, an unstructured N-terminal octadecapeptide which is required for interaction with the membrane protein (II^{Glc}), and the remainder of the molecule. The latter is a compact, thin antiparallel β-sheet sandwich, about 30°A on a side. The active site, His-90, sits in a slight depression in a highly hydrophobic pocket, in close contact with His-75. Neither His is protonated above pH 5, both are required for P-transfer to II^{Glc}, but only H90 for accepting the P from P-HPr. The phosphoryl group forms a hydrogen bond with N-3 of His-75.

There is remarkably little structural change when III^{Glc} is phosphorylated, only shifts of less than one Å in several residues near the phosphorylation site. This sharply contrasts with the behavior of III^{Lac} from Staphylococcus aureus (Deutscher, *et al*, 1982, *Biochemistry*, 21, 4867-4873), which undergoes undergo major structural reorganization when phosphorylated.

Two findings from the structure of the III^{Glc}/glycerol kinase complex stand out. First, both the regulatory and the P-transfer activities of III^{Glc} occur at the region including and surrounding the phosphorylation site. This may explain why phospho-III^{Glc} does not inhibit non-PTS catabolic systems. The second is that III^{Glc} binds at a distance from the active site of glycerol kinase and therefore exerts its inhibitory effect through the structure of the latter protein.

These and future studies may ultimately answer the key question: How does an 18 kDa protein specifically recognized 8 other proteins that do not have a common primary sequence?

Zn(II)-mediated Protein Interactions in *E. coli* Signal Transduction

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IIIglc of the *E. coli* phosphotransferase system interacts with several proteins in its dual roles of phosphocarrier and regulatory subunit. Structures of its complexes with one of the target proteins, glycerol kinase, have been determined in the laboratory of Dr. Jim Remington at the University of Oregon. Recent investigations in his laboratory show that association of these two proteins forms a binding site for Zn(II) with each protein contributing metal ligands. The structure of the intermolecular Zn(II) binding site is identical to that in thermolysin or carboxypeptidase A. Our enzymological studies show that Zn(II) specifically enhances IIIglc inhibition of glycerol kinase, decreasing the Ki from 3 micromolar to 0.2 micromolar. Interestingly, ZnATP is also a substrate for glycerol kinase. A mutation of the IIIglc active site His residue 75, which is also one of the Zn(II) ligands, to Gln has been constructed in the laboratory of Dr. Saul Roseman of the Johns Hopkins University. Our studies show this mutation abolishes Zn(II) enhancement of the inhibition but does not affect the inhibition in the absence of Zn(II). This suggests that the hydrophobic and Zn(II)-coordination components of the interactions between IIIglc and a target protein are independent. These results provide a rationale for the apparent lack of sequence similarity between IIIglc target proteins and suggest an explanation for the relief of IIIglc inhibition by phosphorylation.

THE BACTERIAL PHOSPHOTRANSFERASE SYSTEM: A MULTIFACETED REGULATORY SYSTEM CONTROLLING CARBON AND ENERGY METABOLISM.

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Cytoplasmic inducer generation and the phenomenon of catabolite repression in bacteria appear to be regulated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) by distinct mechanisms in Gram-negative versus Gram-positive bacteria. In Gram-negative bacteria, the free form of the glucose-specific IIA protein of the PTS, which can be phosphorylated on a histidyl residue by PEP and the PTS energy coupling proteins, inhibits non-PTS permease activities, while the phosphorylated form is believed to activate adenylate cyclase. Further, a pleiotropic regulatory protein, FruR, plays a crucial cyclic AMP receptor protein-independent role in catabolite repression of many central enzymes of carbohydrate metabolism. In Gram-positive bacteria, the phosphorylated forms of the energy coupling protein HPr of the PTS, which can be phosphorylated on a seryl residue by ATP and a protein kinase as well as on a histidyl residue by PEP and Enzyme I plays a role in catabolite repression. It functions in conjunction with the DNA binding protein CcpA which is homologous to the enteric bacterial protein FruR. The current status of these PTS-mediated regulatory mechanisms will be evaluated.

POSTER SESSION V (Thursday Afternoon)

G. Begley

Overexpression of *orf162*, a *Klebsiella pneumoniae* gene which may coregulate nitrogen and carbon assimilation via the phosphotransferase system

K. Jahreis

Molecular analysis of two ScrR repressors and of a ScrR-FruR hybrid repressor for sucrose and D-fructose specific regulons from enteric bacteria

H. Kornberg

Utilization of fructose in the presence of glucose by *E. coli*

M. Merrick

A gene linked to the σ factor gene *rpoN* encodes a protein homologous to PTS enzyme IIA proteins

A. Peterkofsky

Regulation of *E. coli* adenylyl cyclase activity by nucleotides

F. Titgemeyer

Regulation of the raffinose permease in *Escherichia coli* by the glucose-specific enzyme IIA of the phosphoenolpyruvate:sugar phosphotransferase system

OVEREXPRESSION OF ORF162, A KLEBSIELLA PNEUMONIAE GENE WHICH MAY COREGULATE NITROGEN AND CARBON ASSIMILATION VIA THE PHOSPHOTRANSFERASE SYSTEM.

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A Klebsiella pneumoniae gene, orf162, has been implicated in the regulation of transcription from promoters requiring the alternate sigma factor, sigma-54, including genes of the global nitrogen regulatory network. The deduced protein sequence of this gene has also been shown to share a consensus sequence for phosphorylation with several proteins of the phosphoenolpyruvate(PEP)-dependent phosphotransferase system (PTS). We have amplified orf162 from K. pneumoniae ssp. aerogenes using the polymerase chain reaction, subcloned it into a temperature-inducible expression vector (pCQV2), and expressed a protein of the appropriate size in Escherichia coli. We have demonstrated PEP-dependent phosphorylation of a protein corresponding in size to ORF162 in extracts of cells harboring this vector (pGSB3). The phosphorylated protein is not observed in extracts either of cells containing the control plasmid, pCQV2, or of a strain of E. coli lacking a functional PTS due to a mutation in ptsH, which encodes the general phosphotransfer protein HPr. Expression of orf162 in the wild type strain is associated with both increased growth on minimal salts media containing 1% glucose and with increased glucose PTS activity (PEP-dependent phosphorylation of glucose or a non-metabolizable glucose analog). These observations suggest a possible role for the PTS in the regulation of nitrogen assimilation via phosphorylation of ORF162.

Molecular analysis of two ScrR repressors and of a ScrR-FruR hybrid repressor for sucrose and D-fructose specific regulons from enteric bacteria

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The *scr* regulon of pUR400 and the chromosomally encoded *scr* regulon of *Klebsiella pneumoniae* KAY2026 both consist of four structural genes: gene *scrA* for an enzyme II of the phosphoenolpyruvate (PEP)-dependend carbohydrate:phosphotransferase system (PTS) specific for sucrose (EIIBC^{scr}); gene *scrB* for a β-D-fructofuranoside fructohydrolase (invertase); gene *scrY* for a sucrose specific outer membrane porin; and gene *scrK* for an ATP-dependent fructokinase (gene order *scrKYAB*) .
Both regulons are negatively controlled by a specific repressor (ScrR). As deduced from the nucleotide sequences, both *scrR* genes encode polypeptides of 334 residues (85.5% identical base pairs, 91.3% identical amino acids), containing an *N*-terminal helix-turn-helix motif. Comparisons with other regulatory proteins revealed 30.6% identical amino acids to FruR, 27.0% to LacI and 28.1% to GalR. Eight *scrR*^{S} super-repressor mutations define the inducer binding domain. The *scr* operator sequences were identified by *in vivo* titration tests of the sucrose repressor and by *in vitro* electrophoretic mobility shift assays. D-fructose, an intracellular product of sucrose transport and hydrolysis, and D-fructose 1-phosphate were shown to be molecular inducers of both *scr* regulons. An active ScrR-FruR hybrid repressor protein was constructed with the *N*-terminal part of the sucrose repressor of *K. pneumoniae* and the *C*-terminal part of the fructose repressor of *Salmonella typhimurium* LT2. Gel retardation assays showed that the hybrid protein bound to *scr*-specific operators, and that D-fructose 1-phosphate, the inducer for FruR, was the only inducer. *In vivo*, neither the operators of the *fru* operon nor the *pps* operon, the natural targets for FruR, were recognized, but the *scr* operators were. These data and the data obtained from the super-repressor alleles confirm previous models on the binding of repressors of the LacI family to their operators.

Lit.

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Utilization of fructose in the presence of glucose by *E.coli.*

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When glucose is added to cultures of *E.coli* growing on fructose, subsequent growth occurs predominantly at the expense of glucose (1). Similarly, glucose or its analogues 2-deoxyglucose or methyl α-D-glucoside inhibit the uptake of fructose by washed cells. This utilization of glucose in preference to fructose can be overcome in at least two ways. One involves a change in the ratio of proteins involved in fructose and glucose utilization, achieved through loss of the repressor of the fructose operon and resulting in constitutive synthesis of the products of that operon (2); the other involves an alteration (designated *cif* (3)) in a component gene of the fructose operon.

In *E.coli,* the fructose operon consists of a promoter region adjacent to genes in the order *fruF fruK* and *fruA.* The genes *fruA* and *fruF* specify components of the fructose-PTS which catalyse, respectively, the PEP-dependent uptake and phosphorylation of fructose to 1-phosphofructose, whereas *fruK* specifies not a component of the PTS but the ATP-dependent phosphorylation of 1-phosphofructose to fructose 1,6-*bis*phosphate.

We here report that *cif* mutants contain 1-phosphofructose kinase, the *fruK*+ product, in much lower activity than do wild-type cells. In contrast, the activities of other components of the fructose operon are slightly elevated but much less so than observed with *fruR* mutants, which lack the repressor. In one such mutant examined, this alteration in 1-phosphofructose kinase activity is associated with a point mutation in the *fruK* gene whereby a CCG has been changed to CTG, resulting in alteration of the deduced amino acid sequence (4) of Pro-181 to Leu-181. It is known (5) that loss of 1-phosphofructose kinase leads to increased synthesis of the *fruA* and *fruF* products; it is possible that impairment, by mutation, of the activity of this kinase may also increase the uptake and phosphorylation of fructose sufficiently to overcome the normal preference for glucose.

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A gene linked to the sigma factor gene *rpoN* encodes a protein homologous to PTS enzyme IIA proteins.

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Bacterial RNA polymerase sigma factors can be divided into two families; a major family homologous to *E.coli* σ^{70}, and a minor family represented by σ^{N} (σ^{54}) encoded by *rpoN*. σ^{N} is required for expression of a wide variety of genes involved in many diverse functions including dicarboxylic acid transport, toluene and xylene catabolism, hydrogenase biosynthesis, pilus production and nitrogen fixation.

The *rpoN* gene has now been sequenced from eleven different bacterial genera and in the majority of cases the gene is flanked by a conserved open-reading frame (ORF) upstream and at least two conserved ORFs downstream. The conservation of these ORFs in a very taxonomically divergent set of microorganisms suggests that their function could be related to that of σ^{N} and mutations in both downstream ORFs affect σ^{N}-dependent transcription (Merrick and Coppard, 1989).

Database searches indicate that the product of the second downstream ORF (ORF3) is highly homologous to enzyme IIA proteins of the PTS system (Merrick, unpublished; Reizer et al., 1992) an observation which raises interesting questions about the possible function of these conserved *rpoN*-linked genes.

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REGULATION OF E. coli ADENYLYL CYCLASE ACTIVITY BY NUCLEOTIDES

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In E. coli, cAMP is synthesized from ATP by the enzyme adenylyl cyclase. The enzyme exists in vivo as a complex with a variety of proteins that confer allosteric regulatory properties on it. Major contributors to the complex include general proteins (Enzyme I, HPr & Enzyme IIA) of the sugar transport system known as the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The adenylyl cyclase regulatory complex shows sigmoid velocity vs. substrate (ATP) curves, which are desensitized to hyperbolic curves in the presence of GTP, suggesting that GTP is an allosteric effector (Peterkofsky & Gollop, Protein Science **2**, 498-505, 1993). At low substrate (ATP) concentrations, GTP and other ribonucleoside triphosphates (UTP and CTP) stimulate adenylyl cyclase activity. Activity stimulation is specific for ribonucleoside triphosphates; GDP & GMP are ineffective. Since the activation by these nucleotides is not cumulative, there appears to be a single site on the adenylyl cyclase complex for nucleotide effectors. Maximal activity stimulation by nucleotides requires the presence of inorganic phosphate (Pi). The allosteric properties of adenylyl cyclase require the organized complex with proteins of the PTS, since activity stimulation by nucleotides is demonstrable in permeable cell preparations, but not in broken cell extracts. The requirement for PTS proteins is further demonstrated by the finding that permeable cell preparations of a PTS deletion strain show adenylyl cyclase activity that is desensitized with respect to nucleotide regulation. HPr of both purified preparations and permeable cell preparations can be photolabeled with Az-[^{32}P]GTP; this labeling is competed out by unlabeled GTP or ATP. In addition, purified HPr was photolabeled with Bz-[^{32}P]GTP. These results suggest that the allosteric binding site for nucleotides in the adenylyl cyclase complex might involve HPr. This is consistent with the finding that the three-dimensional structure of HPr resembles that of nucleoside diphosphate kinase, a protein that binds a variety of ribonucleoside triphosphates. Based on these findings, a model for allosteric regulation of adenylyl cyclase activity is proposed.

Regulation of the Raffinose Permease in *Escherichia coli* by the Glucose-Specific Enzyme IIA of the Phosphoenolpyruvate:Sugar Phosphotransferase System

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Previous studies have identified an allosteric mechanism by which the nonphosphorylated form of the glucose-specific enzyme IIA (IIA^{Glc}) of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) regulates the uptake of non-PTS carbohydrates through direct protein-protein binding. These are the chromosomally-encoded permeases specific for lactose, maltose and melibiose, as well as glycerol kinase. Recent studies have identified residues which when mutated prevent allosteric binding of (IIA^{Glc}) to the target permeases. The three dimensional structure of the *E. coli* glycerol kinase-IIA^{Glc} complex has been solved to a resolution of 2.6 Å.

We here demonstrate that in *E. coli* cells bearing the plasmid-encoded raffinose catabolic system, sensitivity of [^{3}H]raffinose uptake to inhibition by the glucose analogue, methyl α-glucoside, is enhanced by partial thermal inactivation of Enzyme I which is required for IIA^{Glc} phosphorylation. In mutants lacking IIA^{Glc} the sensitivity to inhibition is abolished. The results suggest that the raffinose permease, RafB, which is similar in sequence to the lactose permease, is subject to PTS-mediated regulation. The raffinose system is the first plasmid-born system, which has been shown to be regulated by the PTS.

Demonstration of the sensitivity of the raffinose permease to PTS-mediated regulation allows refinement of the previous, postulated consensus sequence for IIA^{Glc} binding. Comparison of the loop regions of the maltose, lactose and raffinose permeases shows, that they share the motif V(L)-G-A-N-X-S-L(A). Mutagenic studies with RafB will be required to further substantiate the involvement of this region in IIA^{Glc} binding.

Session VI. Polyphosphates and Phosphate Reserves WEDNESDAY 15th

2:25–2:30 CONVENERS: Arthur Kornberg (USA) and Igor S. Kulaev (Russia)

2:30–3:00 Alan H. Goldstein (Dept. Biology, California State Univ.–Los Angeles, USA)
"Solubilization of exogenous phosphates by gram-negative bacteria"

3:00–3:30 Barry L. Wanner (Dept. Biol. Sci., Purdue Univ., USA)
"P_i-regulated genes for the utilization of phosphonates in *Enterobacteriaceae*"

3:30–3:45 (break)

3:45–4:00 Igor S. Kulaev, remarks

4:00–4:30 Arthur Kornberg (Dept. Biochem., Stanford Univ. Sch. Med., USA)
"Multiple functions of inorganic polyphosphate"

4:30–5:00 Hisao Ohtake (Dept. Fermentation Technol., Hiroshima Univ., Japan)
"Genetic engineering of polyphosphate accumulation in *Escherichia coli*"

Solubilization of exogenous phosphates by gram-negative bacteria.

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The metabolic and genetic bases for high efficiency solubilization of poorly soluble calcium phosphates by gram negative bacteria has been identified. We have shown that solubilization is the result of acidification of the periplasmic space by the direct oxidation of glucose (nonphosphorylating oxidation) or other aldose sugars by the quinoprotein glucose dehydrogenase (GDH). Glucose-derived gluconic acid often undergoes one or two additional extracellular oxidations to 2-ketogluconic or 2,5-diketogluconic acid. The enzymes of the direct oxidative pathway are located on the outer face of the cytoplasmic membrane. The products of direct extracellular oxidation may be taken up by specific phosphotransferase or other transport systems. Little is known about the regulation of the genes coding for quinoproteins or how quinoprotein-mediated oxidative metabolism is regulated. Nonphosphorylating oxidation is one of the four major metabolic pathways for glucose (aldose) utilization by bacteria. The quinoprotein GDH controls the unique step in this metabolic pathway. GDH is an aldose dehydrogenase located on the outer face of the cytoplasmic membrane which transfers electrons from aldose sugars directly to the electron transport chain via two electron, two proton oxidations mediated by the cofactor pyrrolo-quinoline-quinone (PQQ). While GDH plays a major role in the regulation of glucose metabolism in bacteria that express the direct oxidation pathway, it has been difficult to assess the utility of this pathway in terms of microbial ecology. Although the gluconic acid production phenotype is widely distrubuted among the gram-negative genera, it has not been possible to identify a major bioenergetic or ecological advantage for this trait. Analysis of the significance of this phenotype is further complicated by differences in regulation of GDH expression that exist between bacterial genera and even species. Virtually every combinatorial form of expression is observed, i.e. constitutive apoGDH expression coupled with inducible PQQ biosynthesis vs constitutive PQQ biosynthesis coupled with inducible biosynthesis of the GDH apoenzyme. In *P. aeruginosa*, GDH is inducible by glucose, gluconate, mannitol and glycerol, whereas in *A. calcoaceticus*, the enzyme is synthesized constitutively. *A. lwoffii* and *E. coli* do not show acid production in the presence of glucose without the addition of exogenous PQQ. Cell free extracts of these two bacteria also show glucose oxidation upon addition of PQQ indicating that GDH apoenzyme was produced constitutively. *A. lwoffi* does not metabolize glucose at all, but nevertheless synthesizes apoGDH constitutively. A missing parameter in these analyses may be that, in many natural ecosystems, Pi is the growth limiting nutrient. Therefore, it is proposed that the GDH pathway provides a significant competitive advantage to bacteria in certain ecosystems by enhancing the availablilty of Pi via acid-mediated dissolution of mineral phosphates.

P_i-regulated Genes for the Utilization of Phosphonates in the *Enterobacteriaceae*

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ABSTRACT

Bacteria such as *Escherichia coli* and *Enterobacter aerogenes* use three kinds of phosphorus (P) sources for growth: inorganic phosphate (P_i), organophosphates, and phosphonates (Pn). When the preferred P source (P_i) is in excess, P_i is taken up by a low affinity P_i transporter (Pit), and genes for high affinity P_i uptake and for use of alternative P sources are repressed. The latter are co-regulated as members of the PHO regulon and include a subset of phosphate-starvation-inducible (*psi*) genes. Although we now know much about both P_i and organophosphate metabolism, by comparison we know relatively little about Pn metabolism. Pn are a large class of organophosphorus molecules that have direct carbon-phosphorus (C-P) bonds in place of the more familiar carbon-oxygen-phosphorus ester bond. Bacteria that use Pn as a P source must be able to break C-P bonds. About six years ago (a few weeks prior to the PHO Meeting in Concarneau), the *psiD* locus was shown to be required for Pn utilization. Subsequent studies in *E. coli* have shown that the *psiD* locus is a fourteen-gene operon that encodes both a binding-protein dependent Pn transporter and a C-P lyase. Subsequent studies in *Enterobacter aerogenes* have shown that it has genes for two separate Pn degradative pathways: a C-P lyase pathway and a phosphonatase pathway. The existence of two pathways (both of which are under PHO regulon control) implies that Pn are an important P source in nature. Studies on Pi-regulated genes for Pn uptake and breakdown will be summarized. Selected recent studies on Pn degradation are described in the citations below.

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Multiple Functions of Inorganic Polyphosphate

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Inorganic polyphosphate (polyP), a long linear polymer of orthophosphates linked by high-energy phosphoanhydride bonds is widely distributed in bacteria, fungi, protozoa, plants and mammals, yet little is known about its cellular functions. Potential roles include: (1) energy source, (2) phosphate reservoir, (3) donor for sugar and adenylate kinases, (4) chelator for divalent cations, (5) buffer for alkaline stress, (6) regulator of transcription, and (7) structural element in competence for DNA entry and transformation.

To identify and examine polyP functions, we have purified enzymes from *E. coli* and yeast responsible for the synthesis and utilization of polyP. The homogeneous enzyme provides a route to the gene which encodes it and a means of modulating its expression from extremes of depletion and overproduction of the enzyme. Phenotypes created by this "reverse genetics" may supply clues to polyP functions in cellular growth, metabolism and development.

We will describe three polyP enzymes from *E. coli*. These include a polyP kinase (PPK) that catalyzes the readily reversible synthesis of polyP from the terminal phosphate of ATP, and two exopolyPases, one of which is identical to the guanosine pentaphosphate hydrolase that generates guanosine tetraphosphate ("the magic spot"), the crucial effector in the stringent response. Phenotypes of *E. coli* mutants lacking PPK suggest important roles for polyP in response to stress and deprivation, as for example, heat lability and survival in the stationary phase of the growth cycle. Two distinctive yeast polyPases have also been identified and will be characterized with respect to substrate specificity and cell-compartment localization.

These purified enzymes also serve as valuable reagents to prepare labeled, well-defined substrates (as with PPK) and to determine (as with various polyPases), the features and abundance of polyP in bacteria, yeast and animal cells.

Genetic Engineering of Polyphosphate Accumulation in *Escherichia coli*

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The removal of phosphorus from sewage is important in the control of eutrophication of surface waters. Activated sludge processes, which are commonly used for treating municipal sewage, are very effective in removing organic pollutants, but they do relatively poorly in removing phosphorus. Low phosphorus contents of sludge bacteria appear to limit the biological phosphorus removal in activated sludge processes.

Many bacteria are known to be capable of accumulating excess phosphorus in the form of polyphosphate (polyP). We report here the genetic improvement of bacterial activity to accumulate polyP using *E. coli* as a test organism. High levels of polyP accumulation were achieved by (i) modifying the genetic regulation and increasing the dosage of the *E. coli* genes encoding polyphosphate kinase (*ppk*), acetate kinase (*ackA*), and the phosphate inducible transport system (*pstS*, *pstC*, *pstA*, and *pstB*) and (ii) genetically inactivating *ppx* encoding exopolyphosphatase. Acetate kinase was employed as an ATP regeneration system for polyphosphate synthesis. The best recombinant strain, which contained both pBC29(*ppk*) and pEP02.2 (*pst* genes) accumulated approximately 10-fold more phosphate (P_i) than did the control strain. The phosphorus content of this recombinant reached a maximum of 16 % on the dry weight basis (49 % as phosphate). About 65 % of the cellular phosphorus was stored as polyP.

A significant amount of polyP was found to be released from the recombinants into the medium. A mobile, soluble pool of polyP was detected in the recombinants by means of ^{31}P NMR. The intensity of polyP signal approximately twice increased when cells were treated with EDTA, a membrane-impermeable chelator, indicating that the soluble pool of polyP existed in the periplasmic space. This is likely to be the source of polyP which was released into the medium. The understanding of the mechanism for forming the soluble pool of polyP in the periplasm appears to be essential for preventing the recombinants from releasing polyP.

POSTER SESSION VI (Thursday Afternoon)

J. Booth
Transport of ATP and phosphate into vacuoles of *Saccharomyces cerevisiae*

C. Dumora
Stereospecificity of 2-aminoethylphosphonate aminotransferase from *Pseudomonas aeruginosa*: a ^{1}H NMR study

G. Dumsday
Phosphate metabolism in *Acinetobacter* strains isolated from sewage

P. Lemos
Phosphorus removal in an activated sludge "fill and draw" system. Polyphosphate and carbon metabolism using *in vivo* NMR

J. Niere
The effects of phosphonate on polyphosphates in oomycete and ascomycete plant pathogens

H. van Veen
P_i efflux in *Acinetobacter johnsonii* 210A: analysis of mechanism and energy coupling

H. van Veen
Regulation of polyphosphate metabolism in *Acinetobacter johnsonii* 210A grown in carbon and phosphate limited continuous cultures

Transport of ATP and Phosphate into
Vacuoles of *Saccharomyces Cerevisiae.*

In the yeast *S. cerevisiae*, polyphosphates are localized primarily to the vacuole (Urech et al. 1978. *Arch. Microbiol.* 116: 275-278). Polyphosphate kinase, which catalyzes the transfer of phosphate from ATP to a polyphosphate chain, has also been reported to be present in yeast vacuoles (Shabalin et al. 1977. *Biochemistry (USSR)* 42 1291-1296). If this enzyme is responsible for the synthesis of vacuolar polyphosphate *in vivo*, there must exist a mechanism by which ATP can cross the vacuolar membrane to gain access to the vacuolar interior. For this reason, we have looked for ATP transport and polyphosphate synthesis in isolated intact vacuoles. We present evidence for the entry of ATP into intact vacuoles, and for the resulting synthesis of short-chain polyphosphates. In addition, we have characterized transport of orthophosphate into these purified vacuoles.

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STEREOSPECIFICITY OF 2-AMINOETHYLPHOSPHONATE AMINOTRANSFERASE FROM PSEUDOMONAS AERUGINOSA : A ^{1}H NMR STUDY.

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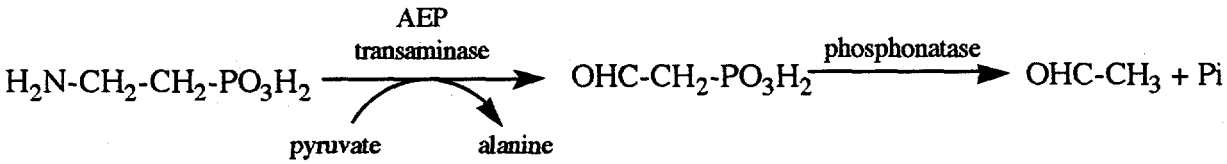
2-Aminoethylphosphonic acid (AEP) is a naturally occurring compound. Its catabolism by *Pseudomonas aeruginosa* involves a double step pathway :

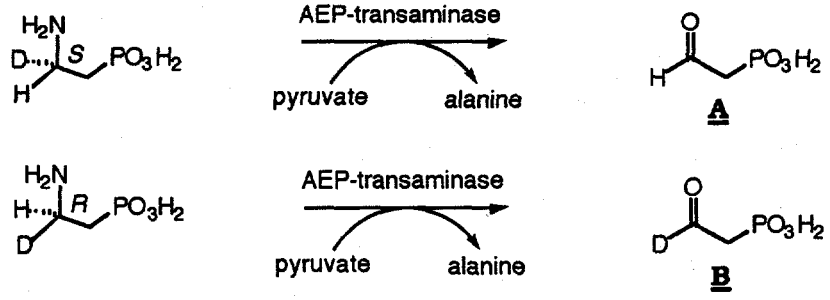


We have characterized these two enzymes (C. Dumora *et al., Eur. J. Biochem.,* 1983, **133**, 119, and *Biochim. Biophys. Acta,* 1989, **997**, 153); we now investigate their stereospecificity.

(*R*) and (*S*)-2-amino-(2-$^2H_1$)ethylphosphonic acids were prepared by F.Hammerschmidt (1988, *Liebigs Ann. Chem.,* 961) to investigate the stereochemical aspect of the initial step of the catalytic process. Indeed, aminotransferase reaction proceeds through the abstraction of a proton from the Schiff-base complex formed between the enzyme bound pyridoxal-P and the substrate. Thus, in the ω-aminotransferase reactions there are two stereochemical possibilities for the proton abstraction.

The purified AEP-aminotransferase was assayed with chiral (*R*)- or (*S*)-AEP in the presence of pyruvate in an aqueous buffer, pH 8, at 303 K. ^{1}H NMR spectra of the reactions were recorded with an AMX-500 Bruker Spectrometer to detect the aldehydic hydrogen of phosphonoacetaldehyde formed A (-CHO, 9.64 ppm) since the deuteriated molecule B (-CDO) did not show any signal at 9.64 ppm.

The data illustrated in the scheme show that the AEP-transaminase catalyses the stereospecific abstraction of the pro-S hydrogen.



Phosphate Metabolism in *Acinetobacter* Strains Isolated from Sewage.

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Acinetobacter spp. have been widely implicated in enhanced biological-removal of phosphate during treatment of sewage in modified activated-sludge systems, due in part, to their ability to synthesize large quantities of intracellular polyphosphate. *Acinetobacter* strains have been isolated from sludge taken from a pilot scale sewage treatment system exhibiting enhanced biological-removal of phosphate. Marked variations were observed in the levels of polyphosphate accumulated by the *Acinetobacter* strains isolated. Mutant strains which do not produce polyphosphate during balanced growth were isolated from the strains which produce large amounts of polyphosphate. These strains have been compared to elucidate the biochemical processes associated with phosphate metabolism in *Acinetobacter*, in particular the mechanism of polyphosphate formation.

The effects of varying different physiological conditions, such as oxygen, on the phosphate pools in the above mentioned *Acinetobacter* strains has been examined.

The specific activity of the polyphosphate kinase, to date the only enzyme reported to be capable of synthesizing polyphosphate, has been determined in the *Acinetobacter* strains after growth in both limiting and excess phosphate. This enabled us to determine if the activity of the polyphosphate kinase is enhanced when polyphosphate is being synthesized. The polyphosphate kinase has also been partially purified and a study of the kinetics has been undertaken to establish if this enzyme is responsible for the large levels of polyphosphate produced by some of the *Acinetobacter* strains.

High extracellular phosphate concentrations promoted the expression of two membrane polypeptides in the *Acinetobacter* isolates which can produce polyphosphate. These polypeptides are produced only to a limited extent in the *Acinetobacter* strains which do not produce polyphosphate. The potential roles of these proteins will be discussed.

PHOSPHORUS REMOVAL IN AN ACTIVATED SLUDGE "FILL AND DRAW" SYSTEM. POLYPHOSPHATE AND CARBON METABOLISM USING *IN VIVO* NMR

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Control of eutrophication, a worldwide water pollution problem, requires removal of phosphorus (P) from effluents by chemical and/or biological means. Activated sludge systems, modified for enhanced P removal, are at present operating in several countries and show considerable advantages over chemical methods [1]. It is well established that enhanced biological P removal depends on the provision of alternate stages in which the activated sludge is subjected to anaerobic and aerobic conditions, respectively. Phosphorus is released from the biomass in the anaerobic stage, and reincorporated during aeration, but these biological processes are still poorly understood. Besides many attractive aspects, biological P removal is known to be relatively inconsistent and subject to a variety of operational problems; therefore, a thorough understanding of the ecology and physiology of the relevant bacteria [2] is essential for achieving a rational optimization of the process.

A laboratory scale "fill and draw" system working at pH 7 with activated sludge obtained from an effluent treatment plant (Beirolas, Portugal), was used in this study. Acetate was supplied as carbon source only during the anaerobic stage. Acetate and phosphorus concentrations as well as biomass were monitored. Several sludge residence times were tested in order to obtain stable operational conditions and efficient P removal; best results were obtained for a residence time of approximately 23 days. Acetate was rapidly consumed during the anaerobic stage and phosphate was released. In vivo NMR studies were performed using sludge from this reactor. Polyhydroxybutyrate and glycogen were detected as internal carbon reserves. The incorporation of carbon-13 labelled acetate into polyhydroxybutyrate was monitored using *in vivo* ^{13}C-NMR. The effect of carbon sources other than acetate on P release was investigated. Synthesis and degradation of polyphosphate was monitored by ^{31}P-NMR.

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The Effects of Phosphonate on Polyphosphates in Oomycete and Ascomycete Plant Pathogens.

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Potassium phosphonate has been shown to be an effective inhibitor of plant diseases caused by the fungal genus Phytophthora. Although the mode of action of phosphonate has yet to be determined, the evidence to date suggests that it disrupts the metabolism of the pathogen to produce both a direct inhibition of growth and a modification of the host response.

The biochemistry of the Phytophthora (class Oomycetes) differs in a number of ways from that of the true fungi. In particular they contain an abundance of short-chain, acid-soluble polyphosphates and relatively little long-chain polyP, the reverse of the situation in Ascomycetes and Basidiomycetes.

^{31}P NMR spectroscopy combined with ^{32}P tracer studies on P. palmivora showed that although the amount and mean chain length of short-chain acid-soluble polyP increased when the level of phosphate supplied was increased from 0.1 to 10 mM, there was no production of long-chain, acid-insoluble polyP.

Extension of the ^{31}P NMR studies to six other members of the genus showed that the above was characteristic of Phytophthora. With the exception of P. infestans, all species examined accumulated more and slightly longer polyP when media phosphate concentration was increased a hundred-fold.

Inhibition of growth by phosphonate resulted in striking increases in polyP and pyrophosphate pool sizes and a decrease in mean polyphosphate chain length by 10-40%. Changes in the sugar and nucleotide phosphate pool sizes were smaller or non-existent. A characteristic of the phosphonate-treated cultures was their continued accumulation of phosphate from the medium at maximum rates even though growth was inhibited. These changes point to a site of phosphonate action which disrupts the regulation of phosphate utilisation in the organism. No such changes were observed when growth was inhibited by treatment with metalaxyl, a fungicide which acts by a mechanism quite different from that proposed for phosphonate.

Acid extracts of phosphonate-treated mycelia also contained isohypophosphate, the phosphate-phosphonate analogue of pyrophosphate. This compound might originate either as a result of cleavage from a nucleotide parent compound in a pyrophosphorylase type reaction or as a result of mitochondrial pyrophosphatase activity.

Phosphonate has also been shown to inhibit growth of Fusarium oxysporum. This ascomycete contained long-chain polyP but very little short-chain polyP. Inhibition of growth by phosphonate did not produce the changes in pyroP or polyP pools observed in Phytophthora species.

Pi EFFLUX IN *ACINETOBACTER JOHNSONII* 210A: ANALYSIS OF MECHANISM AND ENERGY COUPLING

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Acinetobacter johnsonii 210A is able to accumulate an excessive amount of Pi as polyphosphate under aerobic conditions. When oxidative phosphorylation is impaired (e.g., in the absence of oxygen or an electron donor), polyphosphate is degraded and Pi is released into the medium. In cells, two Pi transport systems are present of which one mediates Pi efflux[(1)]. The latter system was characterized in membrane vesicles and proteoliposomes[(2)]. Pi transport is strongly dependent on the presence of divalent metal ions, like Mg^{2+}, Ca^{2+}, Co^{2+}, or Mn^{2+}, which form a soluble, neutral $MeHPO_4$ complex with up to 74 % of the Pi present in the incubation mixture. Pi-dependent uptake of Mg^{2+} and Ca^{2+}, equimolar cotransport of Pi and Ca^{2+}, and inhibition by Mg^{2+} of Ca^{2+} uptake in the presence of Pi, but not of Pi uptake in the presence of Ca^{2+} indicate that a MePi complex is the transported solute. MePi uptake is driven by the proton motive force (interior negative and alkaline). The MePi/proton stoichiometry was close to unity. The transport system mediates efflux and homologous exchange of MePi, but not heterologous exchange of MePi and glycerol-3P or glucose-6P. The exchange and counterflow rates were essentially pH independent while the efflux and uptake rates increased with pH. Efflux was inhibited by the proton motive force, whereas exchange was inhibited by the membrane potential only. These observations are consistent with an ordered mechanism for binding and dissociation of MePi and proton to and from the carrier protein and point to the recycling of a positively charged, protonated carrier protein during exchange. Based on the experimental results, a novel model for the conservation of metabolic energy liberated from the cleavage of polyphosphate is proposed and discussed.

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Regulation of polyphosphate metabolism in *Acinetobacter johnsonii* 210A grown in carbon and phosphate limited continuous cultures

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The response of *Acinetobacter johnsonii* 210A, an organism which is able to accumulate polyphosphate, to low medium phosphate concentrations was investigated in P- or C- (20 mM butyrate) limited chemostat cultures. Under P-deprivation the organism accumulated poly-β-hydroxybutyric acid, the amount of biomass was proportional to the phosphate concentration in the medium and no polyphosphate was formed. When shifting a culture from P- to C-limitation, phosphate was accumulated as polyphosphate. No poly-β-hydroxybutyrate could be detected in these cells. The specific activities of alkaline phosphatase and the phosphate uptake system were induced at residual P_i-concentrations below the detection limit (< 10 μM). Comparison of the maximal uptake rates of the transport systems in low- and high-P_i-grown cells suggests the presence of a constitutive, low-affinity system and an inducible, high-affinity system (1). The specific activities of the enzymes involved in the degradation of polyphosphate, polyphosphatase and polyphospate:AMP phosphotransferase, increased considerably when polyphosphate formation was possible.

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Session VII. Phospholipids and Protein Export THURSDAY 16th

8:45–9:00 CONVENER: Christian Raetz (USA)

9:00–9:30 Eefjan Breukink (Dept. Biochem. Membranes, Utrecht Univ., Netherlands)
"Lipid involvement in protein translocation in the prokaryotic secretion pathway"

9:30–10:00 William Dowhan (Dept. Biochem. & Molec. Biol., Univ. Texas Med. Sch., USA)
"The role of phospholipids in DNA replication in *Escherichia coli*"

10:00–10:30 Frank Pattus (EMBL, Germany)
"Insertion of pore-forming colicins into membranes: an *in vivo* and *in vitro* study"

10:30–10:45 (break)

10:45–11:15 Christian Raetz (Merck Research Labs., Rahway, N.J., USA)
"Bacterial endotoxins: amazing lipids that activate eucaryotic signal transduction"

11:15–11:45 Gunnar von Heijne (Dept. Molec. Biol., Karolinska Inst., Sweden)
"Membrane protein assembly: can protein-lipid interactions explain the 'positive inside' rule?"

11:45–12:15 William Wickner (Dept. Biochem., Dartmouth Med. Sch., USA)
"Studies of catalytic mechanisms with purified *E. coli* preprotein translocase"

Lipid involvement in protein translocation in the prokaryotic secretion pathway

E. Breukink and B. de Kruijff,Department of Biochemistry of Membranes, Center for Biomembranes and Lipid Enzymology, Utrecht University, The Netherlands.

Newly synthesised precursor proteins are exported out of the cytosol of *E.coli* via secretion mechanisms involving in general the signal sequence of the precursor, the Sec-machinery with additional proteinaceous components, acidic membrane phospholipids, ATP and a proton motive force. This lecture will concentrate specifically on the role acidic phospholipids play in this translocation process.
First, the interaction of an essential component of the Sec-machinery, SecA, with phosphatidylglycerol will be discussed. With the help of a biochemical and a biophysical approach it will be shown that the SecA-phosphatidylglycerol interaction and the way it is modulated by nucleotides leads to a view on how SecA can facilitate the insertion of the precursor into the lipid phase of the membrane thereby initiating its translocation.

Second, it will be shown that also in a SecA-independent pathway used by the M13 procoat precursor protein, the negatively charged lipids of the inner membrane play an essential role in efficient insertion and translocation of this protein into the inner membrane. This leads to the suggestion that also signal sequence-lipid interactions play an important role in the translocation pathway.
That in the SecA-dependent pathway signal sequence-phosphatidylglycerol interactions also play an essential role will be shown via experiments using phospholipid biosynthetic mutant strains and a model secretory protein carrying signal sequences with variable charge and hyrdrophobicity.
A translocation model will be presented which incorporates the role of lipid-protein interactions in the process.

THE ROLE OF PHOSPHOLIPIDS IN DNA REPLICATION IN *ESCHERICHIA COLI*. W. Dowhan & W. Xia. Department of Biochemistry and Molecular Biology, The University of Texas Medical School, Houston, TX 77225. (Tel. 713-792-5600, Fax 713-794-4150).

Initiation of DNA replication occurs at the *oriC* locus of the *E. coli* chromosome and requires the organization of a multienzyme complex made up of cytoplasmic proteins including DnaA protein (*dnaA* gene) which initiates replication. The active form of the DnaA protein requires ATP which turns over to tightly-bound ADP resulting in an inactive form with high affinity for the *oriC* locus. Acidic phospholipids (phosphatidylglycerol and cardiolipin) stimulate initiation of DNA replication *in vitro* by facilitating exchange of ADP for ATP to rejuvenate the *oriC*-bound ADP-DnaA protein (Crooke *et al.* (1992) *JBC* **267**: 16779-82). These observations suggest a central role for acidic phospholipids in the organization and function of the DnaA protein-dependent initiation complex.

The committed step for acidic phospholipid synthesis is catalyzed by the *pgsA* gene product. Strains have been constructed (Heacock & Dowhan (1989) *JBC* **264**: 14972-77) in which the level of acidic phospholipids can be systematically varied via IPTG regulation of a ϕ[*lacOP-pgsA*] fusion in a *pgsA* null allele background. When deprived of IPTG, such strains stop growing at a limiting level of 2-3% phosphatidylglycerol, but remain viable for several hours. Such a mutant (strain MDL12) was used to determine whether the lack of acidic phospholipids also limited DnaA protein-dependent initiation at *oriC* as suggested by the above *in vitro* experiments.

Stable DNA replication (SDR) dependent on the RecA protein (*recA* locus) can occur in *rnh* mutants (RNase H protein) at the alternate *oriK* initiation sites and thereby by passing the need for either DnaA protein or the *oriC* locus; SDR is independent of RecA protein in a *lexA* mutant. Therefore, if growth arrest in strain MDL12 is due to limiting acidic phospholipid synthesis necessary for DnaA protein-dependent initiation, then *rnh* mutants should be suppressors of the IPTG dependence of this strain. As predicted, we have found that an *rnh* derivative of strain MDL12 is independent of IPTG for colony formation in a $recA^+$-dependent manner in a $lexA^+$ background and in a $recA^+$-independent manner in a *lexA* background. In addition, the stability of plasmids which rely on an *oriC*-origin for replication was dramatically reduced in strain MDL12 when IPTG was removed from the growth medium while plasmids with a ColE1 origin, which are not dependent on DnaA protein for initiation, were stable in such cells even in the absence of IPTG.

The above results support a critical *in vivo* role for acidic phospholipids in DnaA protein-dependent initiation of DNA replication as first suggested by *in vitro* experiments and parallel the role of acidic phospholipids in organizing the multienzyme complex involved in protein translocation across the inner membrane (Kusters *et al.* (1991) *JBC* **266**: 8659-62).

Insertion of pore-forming colicins into membranes an in vivo and in vitro study.

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Colicins are plasmid-encoded protein antibiotics which kill bacteria closely related to the producing strain (generally *Escherichia coli*). Like many toxins, colicins are composed of structural domains specialized in one of the different steps of the activity *i.e.* targeting, translocation and killing . The major group of colicins is the E1-class, comprised of colicins A, B, E1, Ia, Ib and K which permeabilize the cytoplasmic membrane, thereby destroying the cell's membrane potential These colicins form well defined voltage-gated ion channels in artificial membranes.

Colicins provide a very interesting model system for studying protein insertion into membranes and for studying channel voltage-gating. The 21 kD C-terminal domain of colicin A, isolated after thermolysin digestion, carries the pore-forming activity. It has been crystallized and the structure of the water-soluble form recently solved by x-ray crystallography. This structure can be described as a bundle of ten alpha-helices which are arranged in three layers containing a hydrophobic helical hairpin completely buried within the protein. The present knowledge on the insertion mechanism and membrane bound form of colcin A will be presented.

Negatively charged phospholipids and acidic pH are required for colicins insertion into membranes in vitro. Using HDL11 strain, in which phosphatidylglycerol (PdtGro) content was alterd by varying the PdtGro-phosphate synthase, the effect of the negatively charged phospholid content on the activity of colicins A and N was studied in vivo. Whereas colcin A is sensitive to the level of expression of PdtGro, colicin N does show any dependence. The difference between the behaviour of colicin A and that of colicin N will be discussed.

BACTERIAL ENDOTOXINS: AMAZING LIPIDS THAT ACTIVATE EUCARYOTIC SIGNAL TRANSDUCTION

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Lipid A, the hydrophobic anchor of lipopolysaccharide, is a unique, glucosamine-based phospholipid that makes up the outer monolayer of the outer membrane of gram-negative bacteria (Raetz, C. R. H. *Annu. Rev. Biochem.* **59**, 129-170, 1990). In 1983, we determined the structure of a novel acylated monosaccharide precursor of lipid A, termed lipid X (2,3-diacylglucosamine 1-phosphate). This discovery greatly facilitated the elucidation of the enzymatic pathway for lipid A biosynthesis. So far, most work on lipid A biosynthesis has been carried out with extracts of Escherichia coli. However, the key features of the E. coli pathway are likely to be conserved in diverse gram-negative organisms. The conditional lethality of E. coli mutants defective in UDP-GlcNAc *O*-acyltransferase, the first step of lipid A biosynthesis, demonstrates that lipid A is essential for cell viability and that the pathway we have discovered is not a minor one in E. coli. The cloning and sequencing of key enzymes of lipid A biosynthesis is progressing rapidly and may provide novel insights into lipid A/protein recognition. Access to these enzymes through overexpression on hybrid plasmids greatly facilitates the preparation of novel lipid A analogs. The availability of lipid A substructures and analogs has provided new insights into the mechanisms by which this unique substance activates signal transduction and cytokine synthesis in animal cells.

Membrane Protein Assembly: Can Protein-Lipid Interactions Explain the 'Positive-Inside' Rule?

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The sequence-factors involved in the membrane integration and folding of multi-spanning membrane proteins are becoming increasingly well understood. For these proteins, the final three-dimensional structure seems to depend primarily on two essential features of the nascent chain: the apolar, membrane-spanning α-helices and the distribution of charged residues in the polar connecting loops. Theoretical as well as experimental work has demonstrated a strong correlation between the distribution of positively charged residues and the transmembrane topology - the "positive inside-rule". This suggests a mechanism of insertion and folding where the hydrophobic regions provide the driving force by partitioning into the lipid bilayer, where the distribution of positively charged residues in the polar flanking regions determine which polar loops are translocated and which remain non-translocated, and where the intra-membrane packing of the transmembrane segments gives the final tertiary structure.

The talk will focus on the role of charged residues during *sec*-dependent and *sec*-independent insertion of bacterial inner membrane proteins, and on the possible roles played by negatively charged phospholipids and the membrane potential during the insertion process.

Studies of Catalytic Mechanisms with Purified E. coli Preprotein Translocase

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Preproteins interact with a cascade of chaperones to shield them from aggregation and proteolysis and prevent "tight" folding, which blocks translocation. For proOmpA, the precursor of outer membrane protein A, the final chaperone in this cascade is SecB. The SecB/proOmpA complex binds to the membrane at SecA. SecA forms the peripheral domain of preprotein translocase, and is itself firmly bound by its association with acidic lipids and with the integral domain SecY/E. SecA has separable binding affinities for the SecB chaperone and for the leader and mature domain of the preprotein, assuring accurate sorting of preproteins from newly-made cytosolic proteins. Once a preprotein is bound, SecA is activated to bind (and subsequently hydrolyze) ATP. The energy of ATP binding drives a "loop" of the N-terminus of the preprotein through the membrane. ATP hydrolysis then leads to the release of both the preprotein and the bound ADP and phosphate. The transmembrane preprotein can then undergo a rapid translocation driven by the membrane electrochemical potential. At as yet ill-defined points in the translocation pathway, the translocation intermediate can re-bind to SecA and again have 20-30 residues "stuffed" through the membrane by the energy of ATP binding. Recent studies have shown that the translocase, by continually driving the amino to carboxyl direction translocation, can actively drive the unfolding of the preprotein. The translocating preprotein proceeds through a path in translocase comprised of SecA and SecY subunits. To achieve this, SecA actually "plunges" through the membrane as part of its catalytic cycle, delivering a "loopful" of the preprotein across the membrane.

POSTER SESSION VII (Thursday Afternoon)

R. Keller
Signal sequence mediated precursor lipid interactions as studied by fluorescence spectroscopy and electron spin resonance

M. Nesmeyanova
Role of phospholipids in the secretion of alkaline phosphatase in *E. coli*

M. Roberts
Substrate requirements of bacterial phosphatidylinositol-specific phospholipase C

D. Smith
Use of aminophosphonic acids in the study of phospholipid metabolism in *Tetrahymena*

Signal sequence mediated precursor lipid interactions as studied by fluorescence spectroscopy and electron spin resonance.

Keller, R.C.A.[1], ten Berge, D.[1], Nouwen, N.[1,2], Tommassen, J.[2], Snel, M.[3], Marsh, D.[3] and De Kruijff, B[1].

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The signal sequence plays an essential in protein translocation in the prokaryotic secretion pathway.Genetic and biophysical approaches indicated that both hydrophobicity [1] and the ability to form α-helical structure [2] are important for a functional signal sequence. The anionic phospholipid requirement for efficient translocation [3,4] seems to correlate excellent with the (anionic) phospholipid specificity of membrane insertion [5,6] and α-helix formation [7] of the synthetic signal peptide of PhoE. The study presented here will be focused on whether the signal sequence in the context of the complete precursor also reveales an (spontaneous) ineraction with (anionic) phospholipids. For this purpose we made use of fluorescence techniques like energy transfer and fluorescence quenching and of the electron spin resonance technique (E.S.R.). We made use of mutants of prePhoE in which we have introduced an unique cysteine at different positions in the protein and by means of fluorescent or spin labels, attached to a specific S-H linker, we studied the interaction of the precursor prePhoE with vesicles of different phospholipid composition. This made clear that the signal sequence mediated the interaction between the precursor and phospholipids because mature PhoE demonstrated less interaction than PhoE with the signal sequence attached to it.

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ROLE OF PHOSPHOLIPIDS IN THE SECRETION OF ALKALINE PHOSPHATASE IN E.COLI

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Previously we made a proposal about a significant role of protein-lipid interactions and phospholipid metabolism in structural and metabolic organization of secretory process in bacteria (1,2,3), and obtained, stadying alkaline phosphatase (PhoA) secretion, a number of evidence for it (1-3). They include the presence of PG in the site of interaction of translational complex with membranes at PhoA secretion, correlation of PhoA secretion with the exchange of acid phospholipids and MDO, effects of directed changes in phospholipids by alcohols, polymyxin, temperature shift-down on PhoA secretion, effect of the secretion blocking by protonofore on phospholipid metabolism. In the present work we have studied phospholipid exchange in E.coli strain with a defect in protein secretion resulting from secY mutation, as well as at secretion of mutant PhoA with aminoacid substitutions in processing site and N-terminus of mature PhoA. It was shown that, in E.coli with secY mutation, secretion block correlates at nonpermissive temperature with a 2-fold decrease of PG and MDO, content, PG/CL ratio as well as with the increase of DAG-kinase activity. SecY mutation leads also to temperature sensitivity of phospholipid synthesis and prevents changes in phospholipid metabolism which accompany PhoA secretion. Besides it was shown that substitution of Val for Ala-1 results in secretion of pre-PhoA into periplasm without its processing, which correlates with a 2-fold increase of PG/CL ratio as compared with that in cells secreting wild-type PhoA and mutant proteins with aminoacid substitution of Glu for Gln+4, and double substitution of Ala for Arg+1 and Glu for Gln+4. Stereochemical analysis on molecular models of signal peptide interaction with acid phospholipids showed that this interaction results in induction of α-helical structure of signal peptide and formation of a hydrophobic protein-lipid complex. As this takes place, conditions are created for translocation of the above complex as a whole, which is energetically more favourable than that of its components separately. Possible mechanisms of co-translocation of protein and phospholipid its coupling with phospholipid metabolism, catalyzing role of membrane proteins (possibly Sec) in lipid-protein interaction, as well as role of ATP in secretion are under discussion.

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"Substrate Requirements of Bacterial Phosphatidylinositol-Specific Phospholipase C"

M. F. Roberts, K. A. Lewis, V. R. Garigapati, and C. Zhou

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A series of symmetric short-chain phosphatidylinositols (racemic as well as *D* - and *L*-forms), and 2-O-alkylinositol derivatives of diheptanoyl-PI have been synthesized, characterized, and used to investigate key mechanistic questions about phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis*. This enzyme not only hydrolyzes PI but cleaves membrane GPI-anchored proteins. Key results include the following: (i) bacterial PI-PLC exhibits a 5 to 6-fold "interfacial activation" when its substrate is present in an interface as opposed to existing as a monomer in solution (in fact the similarity to the activation observed with non-specific PLC enzymes, which may have roles in phosphorus scavenging, suggests a similarity in activation mechanisms); (ii) the inositol 2-OH must be free since the enzyme can not hydrolyze diheptanoyl-(2-O-alkyl)-PI (this is most consistent with the formation of cyclic 1,2-inositol phosphate as a necessary step in catalysis); (iii) the size of the modification on the C-2-OH is critical for inhibition of the enzyme; (iv) the inositol ring must have the D-stereochemistry (the L-inositol attached to the lipid moiety is neither a substrate nor an inhibitor); and (v) the presence of noninhibitory L-PI with the D-PI substrate relieves the diacylglycerol product inhibition detected ~30% hydrolysis.

USE OF AMINOPHOSPHONIC ACIDS IN THE STUDY OF PHOSPHOLIPID METABOLISM IN *TETRAHYMENA*. J. Donald Smith, Department of Chemistry, University of Massachusetts Dartmouth, North Dartmouth, MA 02747.

The ciliate protozoan *Tetrahymena thermophila* provides an excellent model system for the examination of eukaryotic phospholipid metabolism since its phospholipids and the pathways for their synthesis are virtually identical to those of higher eukaryotes. We are able to modify the phospholipid composition of the organism *in vivo* by culturing in the presence of one of a number of phosphonic acid-containing analogues of a normal phospholipid head-group, e.g. 2-aminoethylphosphonate, 3-aminopropylphosphonate or N,N,N-trimethyl-2-aminoethylphosphonate. These compounds are incorporated into the phospholipids either in place of or in addition to one of the normal phospholipids. The resulting changes in cell growth, morphology or enzyme activity provide insights on the cellular requirements for specific phospholipids. (Supported in part by NSF Grant DCB-8904979.)

Session VIII. Protein Export, Structural Studies and Folding THURSDAY 16th

7:00–7:15 CONVENERS: Jan Tommassen (Netherlands) and Jonathan Beckwith (USA)

7:15–7:45 Jonathan Beckwith (Dept. Microbiol. & Molec. Genetics, Harvard Med. Sch., USA)
"Pathways of protein disulfide bond formation *in vivo*"

7:45–8:15 Shoji Mizushima (Tokyo Coll. of Pharmacy, Japan)
"Molecular mechanism of protein translocation across the cytoplasmic membrane of *E. coli*"

8:15–8:30 (break)

8:30–9:00 Linda L. Randall (Dept. Biochem. & Biophys., Washington State Univ., USA)
"Recognition of nonnative structure by the molecular chaperone SecB"

9:00–9:30 Jürg P. Rosenbusch (Dept. Microbiol., Univ. Basel, Switzerland)
"The function of phosphoporin at atomic resolution"

9:30–10:00 Jan Tommassen (Dept. Molec. Cell Biol., Utrecht Univ., Netherlands)
"Biogenesis outer membrane porin PhoE of *Escherichia coli*"

Pathways of protein disulfide bond formation in vivo. J. Beckwith, Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115. Tel. 617-432-1920; FAX. 617-738-7664.

In bacteria, disulfide bonds are found mainly in cell envelope proteins and rarely in cytoplasmic proteins. We have found a system responsible for catalyzing disulfide bond formation in cell envelope proteins of *E. coli*. The primary responsible protein is DsbA, a periplasmic protein which can directly promote disulfide bond formation in proteins. DsbA belongs to a family of disulfide bond oxidoreductases that contain a very unstable disulfide bond between two cysteines in the sequence cys-X-Y-cys. Once DsbA has given up its disulfide bond, it is reoxidized by a second protein DsbB. DsbB is a cytoplasmic membrane protein with two periplasmic domains. The four cysteines located in these domains are essential for its activity. The Dsb system is required in *E. coli* for the oxidation of alkaline phosphatase (AP), β-lactamase, OmpA, and the cloned eukaryotic proteins urokinase and tissue plasminogen activator.

The absence of disulfide bonds in proteins in the cytoplasm could be due to the absence of a Dsb-like system and/or to the reducing environment in the cytoplasm. We have sought mutants of *E. coli* that permit disulfide bond formation in the cytoplasm, by selecting for cells capable of activating a cytoplasmically localized AP. At 37°, all mutants obtained have alterations of the *trxB* gene, which codes for thioredoxin reductase. In these mutants, a substantial portion of the cytoplasmic AP contains disulfide bonds. The *trxB* mutation also allowed activation of cytoplasmically localized urokinase. A mutation in *trxA*, which codes for thioredoxin, did not cause this phenotype. The double mutant, *trxA*, *trxB*, exhibited the phenotype of the *trxB* mutant. Our results suggest that thioredoxin reductase plays an important role in maintaining the reducing environment in the cytoplasm as it regards disulfide bond formation.

Molecular Mechanism of Protein Translocation across the Cytoplasmic Membrane of *Eshcerichia coli*

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Roles of individual components comprising the protein translocation machinery of *E. coli* will be discussed based on the following recent findings.

We have succeeded in reconstituting the translocation machinery from SecA, SecY, SecE and phospholipids. By using the reconstitution system, a new membrane protein, p12, was identified as a component of the machinery. Purified p12 stimulated the reconstituted translocation activity more than 20 fold. Anti-p12 antibody inhibited the translocation. The gene encoding p12 was identified and cloned, and the amino acid sequence of p12 was determined. The overproduction of p12 supported the simultaneous overproduction of SecY, suggesting the interaction between p12 and SecY.

SecD was found to be involved in the latter part of the translocation event. Treatment of *E. coli* spheroplasts with anti-SecD antibody resulted in the inhibition of export of periplasmic and outer membrane proteins with concomitant accumulation of their precursor and mature forms in the spheroplasts. The mature proteins accumulating were sensitive to externally added trypsin, suggesting that they have already been exported across the cytoplasmic membrane. We conclude that SecD is involved in the final stage of the translocation reaction, most likely in the release of translocated proteins from the membrane.

The SecF overproduction resulted in the simultaneous overproduction of SecD encoded by the *tac-secD* gene on a plasmid. The overproduction was due to stabilization of SecD with SecF. SecF also stabilized SecY. Since the number of SecF molecule in one *E. coli* cell is only one-tenth of that of SecD or SecY, it is probable that SecF is catalytically involved in the integration of SecD/SecY into the translocation machinery.

RECOGNITION OF NONNATIVE STRUCTURE BY THE MOLECULAR CHAPERONE SECB; Traci B. Topping and Linda L. Randall, Department of Biochemistry and Biophysics, Washington State University, Pullman, WA 99164-4660

Members of the broad family of proteins, termed molecular chaperones, interact with nonnative polypeptides to facilitate a variety of processes including folding of monomers, formation of oligomeric structures, DNA replication, phage assembly and localization of proteins. SecB, a molecular chaperone in *E. coli*, is involved in the efficient export of some proteins from the cytoplasm to the periplasm or into the outer membrane. Our studies of the interaction of the precursor of the periplasmic maltose-binding protein and SecB have lead us to propose a model to account for the intriguing ability of the chaperone to recognize its ligands as nonnative. The precursor is synthesized containing an amino-terminal leader peptide, which is proteolytically removed during export to generate the mature protein. This leader plays an indirect role in the interaction with SecB. It is not specifically recognized, but rather it slows the folding of the polypeptide allowing SecB to bind the central portion of the mature region. SecB, which functions as a tetramer, contains multiple binding sites for flexible regions of polypeptide as well as a site for hydrophobic pitches. A detailed model will be presented.

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Phosphoporin (the product of the *phoE* gene) forms three anion-selective pores per trimer which consist of a vestibulum on the outer surface of the cell, a constriction site in the middle of the channel, and an bulb-shaped exit in open communication with the periplasmic space. The vestibulum is delineated by six loops that form a first filter of solutes passing the pore. Size exclusion and ion selectivity are given by the bottleneck in the channel, with a diameter of about 6x10 Å. The narrowness of the channel is caused by one loop (L3) bending into the channel and carrying two carboxylate groups which are facing a cationic cluster most notable by its thre guanidinium groups on the opposite channel wall. Anion selectivity is provided by a lysyl group which protrudes into the channel. These results are derived from the X-ray structure at 3Å resolution, solved by T. Schirmer (see Cowan et al. (1992) Nature **358**, 727-733), and a site-specific mutation converting the lysyl group into a glu-residue. This change, performed in the group of J. Tommassen, reverses ion selectivity (Bauer et al. (1989) J. Biol. Chem. **264**, 16393-16398). A comparison with matrix porin (the product of the *ompF* gene) allows the following questions to be addressed: i) How is ion selectivity determined (matrix porin is slightly cation selective)? ii) Is there a *bona fide* binding site for anions/phosphate binding in the channel, and what are the alternatives? iii) Channels can open and close as a function of the transmembrane potential: what is the physiological significance of this? iv) What does an open, what a closed channel actually mean? These questions, and experimental approaches to solve them, will be discussed in some details.

Biogenesis outer membrane porin PhoE of Escherichia coli

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PhoE protein is a trimeric, pore-forming outer membrane protein. After its synthesis in the cytoplasm, the protein is translocated across the inner membrane in an non-native, export-competent state. We suppose that it folds at the periplasmic side of the membrane into a ß-barrel. The hydrophobic exterior of this ß-barrel enables insertion into the outer membrane. Trimerization probably occurs after insertion.

We have developed an in vitro system to study the folding, insertion and trimerization of PhoE protein. After its synthesis in an in vitro transcription/translation system, the protein can form a "folded monomer", which is recognized by monoclonal antibodies directed against conformational epitopes in the native trimeric protein. This folded monomer, which migrates faster during SDS-polyacrylamide gel electrophoresis than the denatured protein, could also be detected after in vivo synthesis in pulse-chase experiments. The in vitro trimerization of PhoE was stimulated by the addition of outer membranes. Outer membranes of deep-rough mutants were poorly active, indicating that the core region of the LPS plays an important role during the trimerization process. The trimers obtained in this way were not inserted into the added membranes. Insertion was observed when low amounts of Triton X-100 were added to the in vitro synthesized PhoE, together with the membranes.

The in vitro system was applied to study in detail the effect of mutations that interfere with the biogenesis of PhoE in vivo. The vast majority of bacterial outer membrane proteins contains a phenylalanine as the ultimate C-terminal residue. Substitution of this residue in PhoE had a drastic effect on the biogenesis of the protein. In vitro, the mutations had no effect on the folding of the monomer or on trimerization, but had a drastic effect on the insertion into the outer membranes. Substitution of Gly-144 appeared to affect the folding of the monomers. The introduction of single polar or charged residues at the hydrophobic exterior of the ß-barrel, which faces the lipids in the membrane or is at the monomer-monomer interface, was tolerated. However, the introduction of two polar residues affected strongly the biogenesis in vivo. In vitro, these double mutants showed folding defects.

POSTER SESSION VIII (Thursday Afternoon)

H. Chen
Highly hydrophobic peptide segments are incapable of protein translocation

J. Izard
Polymeric signal sequences discriminate properties for optimizing protein transport

M. Nesmeyanova
Peculiarities of biogenesis and multiple pathways of PhoA secretion into the medium at its oversynthesis by various *E. coli* strains

M. Nesmeyanova
Amino acid substitutions in PhoA affect its biogenesis and secretion in *E. coli*

A. Sukhan
Creation of a model of the structure of OprP

Highly Hydrophobic Peptide Segments Are Incapable of Protein Translocation. H. Chen and D. A. Kendall. Department of Molecular & Cell Biology, Univ. of Connecticut, Storrs, CT 06269

Our laboratory is interested in the requirements for membrane association, translocation, orientation, and stabability of transmembrane segments. As a first step, we used the *E. coli* alkaline phosphotase signal peptide as a model system to study the membrane association and translocation properties of potential transmembrane segments. Previous studies in our laboratory showed that when the wild type signal peptide hydrophobic core region (10 residues) was replaced by 20 leucines the resulting signal peptide was converted to a membrane anchor. The 20L signal peptide was translocated but not cleaved. To explore the possible limits in hydrophobicity of transmembrane segments, two mutants of the *E. coli* alkaline phosphotase were made, 25L and 25LA. These involved replacement of the wild type signal peptide core region with 25 leucines or a 25 leucine and alanine mixture in the ratio of L:A of 3:2, respectively. The 25L mutant is membrane associated but not translocated while the 25LA is almost completely translocated and partially processed by signal peptidase. The reason for the transport defectiveness of the 25L is not clear. One possibility might be that the 25L signal peptide has an unusually high affinity for the membrane and binds to it in a nonspecific and unproductive manner. In contrast, the 25LA binds specifically to the Sec machinery and translocates efficiently. Alternatively, because of the high hydrophobicity of the 25L preprotein, it may have an atypically high affinity for some of the transport machinery. Thus, its release or transfer from one Sec component to the otheris hindered or even halted. The results suggest that, contrary to common expectations, there is an upper limit in hydrophobicity for signal peptides and transmembrane segments and those that are too highly hydrophobic may not be functional. Indeed, the 25L segment is excessively hydrophobic relative to natural transmembrane segments.

Polymeric Signal Sequences Discriminate Properties for Optimizing Protein Transport. J.W. Izard, S.L. Rusch and D.A. Kendall, Dept. Molecular & Cell Biology, Univ. of Connecticut, Storrs, CT 06269.

Two mutants of the *E. coli* alkaline phosphatase signal peptide have been constructed to examine the roles of hydrophobicity and conformation in successful protein transport. These mutants are the 10L mutant, which contains 10 consecutive aliphatic leucine residues in the hydrophobic core region of the signal peptide, and the 10F mutant containing 10 consecutive aromatic phenylalanine residues. Both mutants, by some criteria, function more efficiently in transport than the wild type. In a pulse-chase study, no precursor is evident at the earliest time point for either of these mutants. Although similar with respect to rapid processing, these two mutants differ in how cellular inhibitors affect their transport and how these two mutants affect the transport of other secretory proteins. The data reveal functional differences in three interrelated aspects of transport: SecA interactions, dependence on the protonmotive force and competition for the export pathway. These functional differences are correlated with structural differences in polyleucine and polyphenylalanine, and suggest that requirements for both hydrophobicity and overall conformation are overlayed for at least one key step of transport, such as SecA binding.

PECULIARITIES OF BIOGENESIS AND MULTIPLE PATHWAYS OF PhoA SECRETION INTO THE MEDIUM AT ITS OVERSYNTHESIS BY VARIOUS E.COLI STRAINS

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The study of dynamics of production, localization, isoenzyme spectrum and immunoblotting of PhoA in various E.coli strains showed alterations in PhoA biogenesis at its oversynthesis encoded by phoA gene constituent of multicopy plasmids. They involve accumulation of intermediate forms of the enzyme corresponding to different stages of its posttranslational modification as well as alternative localization of PhoA. Cytoplasm of overproducers was found to contain great quantities of pre-PhoA as insoluble aggregates. Mature PhoA was found as three isoenzymes with a greater, as compared with the initial strains, quantity of metazyme I and III, as well as active PhoA multimers. Under conditions of E.coli cells intactness, mature PhoA was found not only in periplasm but also in the culture medium (50 to 90% of the total PhoA content).

Multiple pathways of PhoA secretion into the medium depending on the nature of a strain transformed by phoA plasmid were revealed. In E.coli C90 with PhoA constitutive synthesis we obtained nonspecific secretion of PhoA and other periplasmic proteins (similar to secretion of Colicins or that in lky mutants) which correlates with one-order increase of lysophospholipid content in membranes, as well as with membrane fragmentation. In E.coli K12802, selective secretion of PhoA with the help of outer membrane vesicles was revealed. Isolated vesicles contain major outer membrane proteins, LPS, but no lipoprotein. In E.coli DHI, selective secretion is accompanied neither by formation of vesicles nor by the above changes in chemical composition of the envelope. It correlates with the increase of the ratio of acid phospholipids CL/PG and some peculiarities of envelope ultrastructure revealed by electron microscopy, in particular, the large extent of contact areas of cytoplasmics and outer membranes.

Accumulation of intermediate forms of PhoA under its oversynthesis is due to a restricted number of membrane translocation sites, and probably the absence of translation block for secreted proteins in bacteria. Variety of secretory pathways is obviously determined by peculiarities of the producer envelope and correlates with alterations in its composition and structure specific for each strain.

AMINOACID SUBSTITUTIONS IN PhoA AFFECT ITS BIOGENESIS AND SECRETION IN E.COLI

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To reveal the role of PhoA primary structure in PhoA secretion and biogenesis, we have studied the effect of aminoacid substitutions in processing site and in N-terminus of mature PhoA on these processes. It was shown that substitution of Val for Ala-1 prevents protein processing in vivo and in vitro, but has no effect on mutant pre-PhoA translocation into periplasm where it was found by immunoblotting and analysis of enzyme activity. Results show that processing is not a limiting stage of translocation, and signal peptide does not prevent formation of an active macromolecule in periplasm. Similar results were obtained with substitution of Pro for Arg+1, though this substitution is not localized in the recognition site of signal peptidase. Substitution of Gln+4 for Glu+4, increasing the positive charge of N-terminus, as well as double substitution of Ala+1 for Arg+1 and Gln+4 for Glu+4, removing N-terminal arginine without changing N-terminal charge, do not result in significant changes of processing in vivo. Double substitution enhances, however, the processing velocity for isolated pre-PhoA in vitro with signal peptidase, as well as during pre-PhoA translocation into membrane vesicles. Substitutions of N-terminal PhoA arginine by various aminoacids prevent post-translocational modification and formation of isoenzymes, which reveals arginine specificity of modifying periplasmic protease. The above mutations have no effect on biogenesis peculiarities which are caused by PhoA oversynthesis encoded by mutant phoA genes constituent of multicopy plasmids. Precursors of mutant proteins are accumulated in cytoplasm as insoluble aggregates, and soluble mutant proteins are localized not only in periplasm, but also in culture medium.

CREATION OF A MODEL OF THE STRUCTURE OF OPR P. A. Sukhan,R. Siehnel and R.E.W. Hancock. Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada.

Upon growth in low concentrations of phosphate (< 0.2mM) *P.æruginosa* expresses a major outer membrane protein (OprP) with a monomer molecular mass of 45,000 daltons.The purified protein was found to form SDS resistant trimers which incorporated into lipid bilayer membranes to form anion-specific channels. Chemical modification studies have demonstrated that OprP possesses an anion-binding site within the channel involving the ε-amino groups of symmetrically placed lysine residues and that this binding site accounts for OprP's 80-100 fold higher affinity for phosphate ions over chloride or other anions.

The structure of OprP is thought to resemble that of PhoE, the analogous phosphate inducible porin in *E.coli*. The current model of PhoE describes the monomer as forming a 16-stranded anti-parallel β-sheet structure which wraps around to form a barrel, enclosing a central pore. The loops between the strands are all short on one side while they are longer on the other side. The longer loops face the external surface of the cell and are important in defining the exclusion limit of the pore. Insertional mutagenesis of PhoE has been used to determine which loops are surface exposed as well as which are involved in maintaining the specific dimensions of the channel. Loops in PhoE which have been determined by this method are 100% consistent with the crystal structure.

The purpose of the current study was to create a model of the structure of OprP using various genetic, immunological and biochemical techniques. The *oprP* gene was mutagenized to remove existing PstI sites without altering the amino acid sequence and cloned into the phagemid pTZ19U. A gel purified kanamycin resistance cartridge isolated from the plasmid pUC4KAPA by cutting with the blunt end cutting restriction enzyme HinCII was cloned into *oprP* which had been previously cut at one of a number of sites by partial digestion with blunt end cutting enzymes. The plasmids were transformed into competent *E.coli* cells, and after selecting for kanamycin resistant colonies, plasmids were isolated by alkaline lysis. The vectors were then digested with PstI, releasing the kanamycin cartridge, religated, transformed into *E.coli* and checked for expression of OprP. The resulting plasmids, containing 12 base pair inserts, were sequenced by the di-deoxy terminator method to define those sites permissive for insertion of four extra amino acids. Mutant forms of OprP created by this procedure were purified by FPLC and assessed for alterations in porin forming ability by the black lipid bilayer method.

It is hoped that by combining the results of the current work with those obtained from ongoing crystallographic studies an accurate model of the structure of OprP will soon be available.

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Session IX. Signal Transduction and Phosphoproteins FRIDAY 17th

8:45–9:00 CONVENERS: Ann Stock (USA) and Hideo Shinagawa (Japan)

9:00–9:30 Hideo Shinagawa (Res. Inst. for Microbial Diseases, Osaka Univ., Japan)
"Signal transduction in the phosphate regulon of *Escherichia coli*: dual functions of PhoR as a protein kinase and a protein phosphatase"

9:30–10:00 Shiro Iuchi (Dept. Microbiol. & Molec. Genetics, Harvard Med. Sch., USA)
"Signal transduction in the Arc system for aerobic metabolism in *Escherichia coli*"

10:00–10:30 Austin Newton (Dept. Molec. Biol., Princeton Univ., USA)
"Role of histidine protein kinases and response regulators in bacterial cell division and polar morphogenesis"

10:30–10:45 (break)

10:45–11:15 Alexander J. Ninfa (Dept. Biochem., Wayne State Univ. Sch. Med., USA)
"Regulation of bacterial nitrogen assimilation by the two-component system NRI and NRII (NtrC and NtrB)"

11:15–11:45 Ann Stock (Ctr. for Adv. Biotechnol. & Med., Piscataway, N.J., USA)
"Structure and function of phosphorylated response regulators in bacteria"

11:45–12:15 Andrew Wright (Dept. Molec. Biol., Tufts Univ., USA)
"Modulation of activity of a regulatory protein by PTS-mediated phosphorylation and dephosphorylation controls *bgl* operon expression"

Signal Transduction in the Phosphate Regulon of *Escherichia coli;* Dual Functions of PhoR as a Protein Kinase and a Protein Phosphatase

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The genes in the phosphateregulon of *E. coli* are transcriptionally activated by the phosphorylated PhoB protein when the cell is starved for phosphate. PhoR protein mediates phosphorylation and dephosphorylation of PhoB, responding to limiting and excess phosphate conditions, respectively. The environmental levels of Pi is monitored by periplasmic phosphate binding protein encoded by *pstS* and the signal for excess phosphate is transmitted across the cytoplamic membrane by the products of *pstC, pstA, pstB* and *phoU* to PhoR. Activation of PhoB by phosphorylation can be achieved by alternative molecular mechanisms (cross-talk) in the absence of PhoR. In *phoR* mutants, PhoB is activated by phosphorylation mediated by PhoM, which is a structural and functional homologue of PhoR. In *phoR-phoM* double mutants, activation of PhoB can be achieved by elevated levels of acetylphosphate, which is a direct donor of phosphate moiety to PhoB.

To elucidate the mechanisms of the functional interconversion and the functional domains of PhoR, which is composed of 431 amino acids, we attempted to isolate various types of *phoR* mutants. The PhoR1084 and PhoR1159 proteins that lack the 83 and 158 N-terminal amino acids, respectively, behaved as weakly consitutive activator of PhoB in vivo, while PhoR1263, lacking the 262 N-terminal amino acids including a putative site of autophosphorylation, His 213, was deficient in both functions. Three kinds of *phoR* point mutants were isolated by treating the cloned *phoR* gene with hydroxlamine. Mutants locked in the activator form were isolated. They had following alterations; R204W, T217M, P218S, T220N, and Q371K. Mutants locked in the inactivator form were also isolated. Three independent mutants had the same alteration in H213Y, which is a putative autophosphorylation site of PhoR. A null phenotype PhoR mutant, PhoR (G388E) became inactivator type upon truncation of its 83 N-terminal amino acids.

From these analyses, it was inferred that amino terminal half of PhoR, which includes two transmembrane regions, plays a role in cotrolling the functional conversion of PhoR between protein-kinase and phosphatase by interacting with the carboxyl half, which contains catalytic domains for the kinase and phosphatase. The kinase and phosphatase domains overlap. His 213 is likely to be the auto-phosphorylation site, which is essential for the kinase activity but dispensable for the phosphatase activity.

Signal transduction in the Arc system for aerobic metabolism in *Escherichia coli*. S. Iuchi and E. C. C. Lin. Dept. Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston MA 02115. Tel: 617 432-1926. FAX: 617 738-7664. Email: siuchi@warren.med.harvard.edu

ArcB (encoded at min 69.5) is a transmembrane sensor of a two-component system. ArcB is atypically large: on its N-terminal side is a sensor structure with the expected transmitter module containing the canonical His residue (His^{292}), but on its C-terminal side there is an unexpected regulator structure with a receiver module containing the canonical "acid pocket" constituted by Glu^{532}, Asp^{533}, and Asp^{576}. It appears that upon stimulation by anaerobiosis ArcB undergoes autophosphorylation first at the conserved His^{292} in the transmitter module. This phosphoryl group is then transferred to Asp^{576} in the receiver module. The free His^{292} then undergoes another round of autophosphorylation. This time the phosphoryl group is transferred to cognate regulator ArcA (encoded at min 0). Phosphorylation of ArcA renders it functional in the regulation of gene expression. With a few exceptions, ArcA-P acts as a negative regulator of transcription of operons encoding enzymes involved in aerobic metabolism, including certain primary dehydrogenases of the flavoprotein class, members of the TCA cycle and the glyoxylate shunt, member(s) of the pathways for fatty acid degradation, and cytochrome *o*.

Results obtained by both mutational analysis of the receiver module of ArcB and in vitro phosphorylation analysis of ArcB suggest that the ArcB receiver module is involved in regulation of transphosphorylation from the His^{292}-P to ArcA.

Role of histidine protein kinases and response regulators in bacterial cell division and polar morphogenesis. Todd Lane, Greg Hecht, Noriko Ohta, and Austin Newton, Department of Molecular Biology, Princeton University, Princeton, NJ 08544; Tel. 609/258-3854; FAX 609/258-6175; Email ANEWTON@PUCC.

Temporal and spatial cues required for the sequence of developmental events leading to formation of the new swarmer cell and its subsequent differentiation into a stalked cell in *C. crescentus* are provided steps in by the underlying cell division cycle, including chromosome replication and cell division. A pseudorversion analysis of pleiotropic *pleC* mutants defective in motility and stalk formation has identified cold sensitive suppressors that map to three new cell division genes, *divJ*, *divK*, and *divL* (Sommer & Newton Genetics, **129**, 125, 1991). Nucleotide sequence analysis suggested that the products of these genes are members of a signal transduction pathway coupling polar morphogenesis to events in the cell cycle. The *divJ* gene encodes a predicted protein with sequence homology to a family of bacterial sensor proteins with histidine kinase activity (Ohta *et al.*, PNAS, **89**, 10297, 1992), and we have now shown that the purified kinase domain of the DivJ protein is autophosphorylated *in vitro*. Ely and co-workers have demonstrated that *pleC* also encodes a histidine protein kinase. The *divK* gene encodes a 125 residue polypeptide which is homologous to the *Salmonella* response regulator CheY. Two observation suggest that DivK is the cognate response regulatory of the PleC kinase: (i) *divK* mutations act as a bypass suppressors of *pleC* mutations and (ii) the *divK* gene is transcribed during a restricted period of the cell cycle that corresponds closely to the time at which the *pleC* gene is required for proper development. Recently we have demonstrated and partially characterized the PleC mediated phosphorylation of DivK *in vitro*. We propose that phospho-transfer from the membrane-associated PleC protein kinase to the DivK response regulator constitutes one facet of a complex signal transduction pathway that, along with DivJ and perhaps other uncharacterized gene products like DivL, plays a central role in coupling developmental events to progress through the *Caulobacter* cell division cycle.

Regulation of Bacterial Nitrogen Assimilation by the Two-Component System NRI and NRII (NtrC and NtrB).

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Transcriptional activation of the bacterial Ntr regulon in response to nitrogen limitation requires the phosphorylated form of the response regulator protein NRI (NtrC). The phosphorylation and dephosphorylation of NRI is controlled by the kinase/phosphatase protein NRII. Previous work has indicated that NRII becomes autophosphorylated at residue His139 upon incubation with ATP, and this phosphoryl moiety is transfered to NRI, converting NRI to the form able to activate transcription from nitrogen-regulated promoters. We will present recent data on 4 aspects of this regulation:

The autophosphorylation of NRII proceeds by trans-phosphorylation of the subunits within the dimeric NRII protein. We will describe a method that results in the reversable dissociation of NRII dimers, permitting the formation of hybrid dimers *in vitro* from seperately purified proteins. We show data indicating that within such dimers, ATP bound to one subunit phosphorylates the target site on the other subunit in the dimer. Our data indicate that the autophosphorylation necessarily proceeds by this mechanism, that is, intermolecular trans-phosphorylation does not occur and monomers do not phosphorylate themselves within the dimeric NRII.

We have systematically altered the most highly conserved residues within the kinase/phosphatase domain, and characterized the altered proteins in intact cells. Our data indicate that the highly conserved residues have no effect on the negative regulatory function (phosphatase activity) of NRII, but affect to various extents the positive regulatory function (kinase activity). In addition, we have purified several of the altered proteins and characterizaed them biochemically. Our data confirm earlier results suggesting that the kinase and phosphatase activities of NRII are genetically seperable.

In intact cells, the phosphatase activity of NRII is elicited upon interaction of NRII with the signal transduction protein PII. We developed an improved method for the purification of PII, and demonstrate that PII so prepared is active in eliciting the NRII-dependent dephosphorylation of NRI~P. The interaction of PII with mutant forms of NRII will be presented. In addition, the crystallization of PII and our preliminary structural characterization of the protein will be presented.

In addition to phosphorylation by transfer of the phosphoryl moiety from NRII~P, NRI can be phosphorylated by acetyl phosphate *in vitro* and *in vivo*. We demonstrate that NRI~P so formed is identical to that formed by transfer of phosphoryl groups from NRII~P. Furthermore, we show that the acetyl phosphate in intact cells has a role in the nitrogen-regulation of transcription of *glnA* (encoding glutamine synthetase) in cells lacking NRII.

STRUCTURE AND FUNCTION OF PHOSPHORYLATED RESPONSE REGULATORS IN BACTERIA

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The bacterial chemotaxis response regulator protein, CheY, is a representative member of a family of over 60 signal transduction proteins that share a common structure and mechanism of action. The conserved domain of these response regulator proteins consists of an amino-terminal switch domain that is activated by phosphorylation and is responsible for regulating associated or downstream effector functions. The switch domain itself catalyzes the phosphotransfer and dephosphorylation reactions that result in activation or inactivation of the response regulator. CheY, which regulates the direction of flagellar rotation, is the only response regulator protein for which a three dimensional structure has been determined. Structure/function analysis of CheY serves as a model for understanding the molecular basis of activity of the family of response regulator proteins.

The active site of CheY binds a divalent metal ion that is essential for catalysis of phosphotransfer and dephosphorylation. We have determined conditions that allow for high occupancy of divalent metal ion in the active site of crystalline CheY, and have determined the X-ray structure of *Salmonella typhimurium* CheY with Mg^{2+} bound at the active site to 1.8 Å resolution. The Mg^{2+} exhibits classic octahedral coordination to two active site carboxylate side chains, a backbone carbonyl oxygen, and water molecules. While the overall structures of metal-bound and metal-free CheY are similar, significant rearrangements occur within the active site involving the three most highly conserved residues of the response regulator family. Conservation of the cluster of carboxylate side chains at the active site of response regulator domains can be rationalized in terms of their role in coordinating the catalytically essential divalent cation. The Mg^{2+} coordination geometry provides insights to the mechanism of phosphoryl transfer catalyzed by the response regulator domains.

Modulation of Activity of a Regulatory Protein by PTS-Mediated Phosphorylation and Dephosphorylation Controls *bgl* Operon Expression..

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Department of Molecular Biology, Tufts Medical School, Boston, Ma. 02111

The *bgl* operon of E.coli K-12 is regulated by a novel two-component system which consists of a sensory protein, BglF, present in the cytoplasmic membrane and a regulatory protein, BglG, present in the cytoplasm. The sensor is a phosphotransferase system (PTS) transport protein which controls the activity of the regulatory protein according to substrate availability. The regulator is an RNA binding protein which controls operon expression by transcriptional antitermination. In the absence of substrate, BglF phosphorylates BglG thus inactivating it. In the presence of substrate, BglF removes the phosphate from BglG, thus allowing it to function as an antiterminator. We will show that BglG exists in two forms, one being a phosphorylated monomer which is inactive, the other being a non-phosphorylated dimer which is active. Thus dimerization is modulated by reversible phosphorylation. The properties of dominant negative mutants of BglG support a model for BglG action in which there is a single RNA binding site per dimer which is presumably formed in the process of dimerization. The relationship of this site to the site of phosphorylation and to the RNA recognition sequence will be discussed.

Analagous regulatory systems which share considerable homology with the bgl system exist in Bacillus species and in other Gram negative bacteria. Thus this mode of regulation, though novel at present, is likely to be quite widespread in bacteria. Whether such systems are also present in eukaryotic cells remains to be seen.

POSTER SESSION IX (Thursday Afternoon)

S. Austin

Autophosphorylation and ATP hydrolysis by a mutant NTRC protein defective in transcriptional activation

R. Libby

Phosphorolytic error correction during transcription

Autophosphorylation and ATP hydrolysis by a mutant NTRC protein defective in transcriptional activation.

SARA AUSTIN AND RAY DIXON

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The nitrogen regulatory protein NTRC is a member of the family of response regulator proteins whose activity is controlled by phosphorylation in response to environmental stimuli. The activity of NTRC is regulated through phosphorylation and dephosphorylation of its N-terminal domain by the histidine protein kinase NTRB. The phosphorylated form of NTRC activates transcription by catalysing the isomerisation of closed promoter complexes between σ^{54}-RNA polymerase and promoter DNA, to open complexes in a reaction which requires ATP hydrolysis. Consistent with this is the presence of a putative nucleotide binding site in the conserved central domain of σ54-dependent activator proteins.

A mutant NTRC protein with a single amino acid substitution, S170A, in the phosphate binding loop of the putative ATP-binding site cannot catalyse open complex formation but is capable of stabilising the interaction of σ^{54} holoenzyme with the *nifL* promoter (Austin *et al.*, 1991). Unlike wild-type NTRC the mutant protein possesses a constitutive ATPase activity which is not stimulated by phosphorylation or DNA binding. S170A NTRC was also found to autophosphorylate on its N-terminal domain in the absence of NTRB, in contrast to the wild-type protein. Another substitution in the same residue, S170L, also gives rise to a protein which is inactive as a transcriptional activator. However, unlike the S170A protein, S170L does not autophosphorylate or hydrolyse ATP. Thus, although the S170A protein is capable of interacting with σ^{54} holoenzyme at the promoter and hydrolysing ATP, it cannot couple this to open complex formation. Substitution of the more bulky leucine residue instead of alanine at position 170 presumably impedes the ability of the protein to hydrolyse ATP.

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Phosphorolytic error correction during transcription

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Escherichia coli DNA-directed RNA polymerase is shown to contain a novel phosphorolytic error correction activity which removes erroneous nucleotides, as rNDPs, from the 3′-end of the growing transcript. The activity we describe is biochemically similar to polynucleotide phosphorylase (PNP), yet in contrast to PNP is completely dependent on the presence of Mn^{++}. We demonstrate that the activity, which is mediated by P_i, is preferential for the presence of an incorrectly incorporated nucleotide at the leading 3′-end of the transcript. These findings suggest the possibility that RNA phorphorolysis may play a critical role in the process of transcriptional proofreading.

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