Inorganic Polyphosphate: Essential for Growth and Survival

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Abstract

Inorganic polyphosphate (Poly P) is a polymer of tens to hundreds of phosphate residues linked by "high-energy" phosphoanhydride bonds as in ATP. Found in abundance in all cells in nature, it is unique in its likely role in the origin and survival of species. Here, we present extensive evidence that the remarkable properties of Poly P as a polyanion have made it suited for a crucial role in the emergence of cells on earth. Beyond that, Poly P has proved in a variety of ways to be essential for growth of cells, their responses to stresses and stringencies, and the virulence of pathogens. In this review, we pay particular attention to the enzyme, polyphosphate kinase 1 (Poly P kinase 1 or PPK1), responsible for Poly P synthesis and highly conserved in many bacterial species, including 20 or more of the major pathogens. Mutants lacking PPK1 are defective in motility, quorum sensing, biofilm formation, and virulence. Structural studies are cited that reveal the conserved ATP-binding site of PPK1 at atomic resolution and reveal that the site can be blocked with minute concentrations of designed inhibitors. Another widely conserved enzyme is PPK2, which has distinctive kinetic properties and is also implicated in the virulence of some pathogens. Thus, these enzymes, absent in yeast and animals, are novel attractive targets for treatment of many microbial diseases. Still another enzyme featured in this review is one discovered in *Dictyostelium discoideum* that becomes an actin-like fiber concurrent with the synthesis, step by step, of a Poly P chain made from ATP. The Poly P–actin fiber complex, localized in the cell, lengthens and recedes in response to metabolic signals. Homologs of DdPPK2 are found in pathogenic protozoa and in the alga *Chlamydomonas*. Beyond the immediate relevance of Poly P as a target for anti-infective drugs, a large variety of cellular operations that rely on Poly P will be considered.

Contents

INTRODUCTION

Inorganic polyphosphate (Poly P) (**Figure 1**) is a linear polymer of phosphate residues of lengths from tens to many hundreds, linked by the phosphoanhydride bonds found in ATP. Occurring in volcanoes and deep-oceanic steam vents, Poly P is produced simply by dehydration of phosphate rock at high temperature and was likely present on earth prebiotically.

In speculations about the origins of life, one can imagine the advantages gained by a vesicle, which incorporated Poly P and thus became the source of our phosphate world. Poly P is stable over a wide range of temperatures, pH measurements, and oxidants. It can serve as a source of energy; as a phosphorylating agent for alcohols, including sugars, nucleosides, and proteins; and as a means of activating the precursors of fatty acids, phospholipids, polypeptides, and nucleic acids.

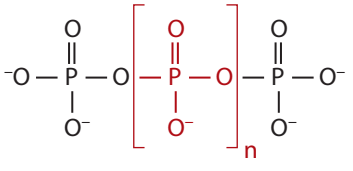

Figure 1

Inorganic polyphosphate (Poly P). Value of n for the $[PO_3^-]$ residue may vary from tens to many hundreds depending on the cellular locations and the metabolic state.

Observed as metachromatically stained "volutin granules" in microbes a century ago, these were shown much later to be simply Poly P and subsequently found abundant in every cell in nature. Our interest in Poly P was spurred initially by its possible relevance to energy metabolism at a time before mitochondria and the mechanism of ATP biosynthesis were discovered (1, 2). Some 40 years later, our interest in Poly P was renewed when it was identified as part of a calcium-Poly P complex that renders *Escherichia coli* competent to take up plasmid DNA (3).

Our approach has been to identify the enzymes responsible for the synthesis and utilization of Poly P and the genes that encode them. What has become clear is that Poly P is chemically suited for a proposed role in the origin and survival of species (4). As an intracellular ion, phosphate levels are maintained in a narrow range near 10 mM surrounded by extracellular fluids that may vary by six orders of magnitude, from micromolar to molar. As a multivalent anion, it is favored as the means for the essential cations of Ca, Mg, Mn, Fe, and Co to perform their biologic roles. Most recently, Poly P was shown to complex with actin-like fibers in cells (5). This property of Poly P can be used to localize it in cells, responding to a variety of metabolic and environmental signals.

For more widespread research on Poly P, improvements in assay methods are needed. In particular, recognition of the several distinctive size groups of Poly P chains makes it clear that our knowledge of their biosynthesis, cellular location, and functions is inadequate. Also needed is an understanding of the masking of the ends of some Poly P chains that make them resistant to the hydrolytic action of exopolyPases. This review focuses on the role of Poly P in pathogenicity with special emphasis on tuberculosis, cystic fibrosis, and enteric diseases. Recent in-depth studies, including structural studies on enzymes that utilize/hydrolyze Poly P like NAD kinase, exopolyphosphatase (exopolyPases PPX1 and PPX2 of *Escherichia coli* and scPPX1 of *Saccharomyces cerevisiae*), and endopolyPase (PNN1), are highlighted. A new group of Poly P-synthesizing enzymes, PPK2, which is present in a variety of prokaryotic and eukaryotic organisms, is introduced. This review also describes the involvement of Poly P in protein degradation, blood clotting, and bone regeneration.

In previous reviews of Poly P, we noted the disparity between the increasing number of essential metabolic functions displayed by Poly P and the sparse attention given to the subject (6–8). Not only has Poly P been absent from texts on biology and chemistry but, even when noticed, was dismissed as a "molecular fossil." At the conclusion of this review, we reflect again on how such an attitude can prevail and discuss the social, political, and economic forces that make Poly P a relatively lonely outpost in the frontiers of science.

ANALYSIS OF CELLULAR POLY P: AMOUNTS AND CHAIN LENGTHS

For more widespread research on Poly P, improvements in assay methods are needed. Among the reasons for the neglect of Poly P research has been the lack of sensitive and facile analytical methods to assess its concentration in biological sources. Enzyme-based assays have to take into account the presence of multiple forms of Poly P, since the enzymes used as reagents are chain length specific. Poly P with masked ends requires modification before assaying with exopolyPases.

Enzymatic and Ion Exchange Methods

One assay requires labeling of cells with $^{32}P_i$, separation of Poly P, and the action of a yeast exopolyPase (scPPX1) to hydrolyze the separated Poly P to phosphate (P_i) (measured either by thin-layer chromatography or by phosphoimager) (9). Another enzyme-based assay uses the reverse reaction of Poly P kinase 1 (PPK1) to measure conversion of ADP to ATP by Poly P in a specific and sensitive luciferase method (10). Chain-length specificity of these assays below 60 P_i residues has not been analyzed in detail.

Ion exchange chromatography equipped with an online hydroxide eluent generator was used to analyze Poly P up to 50 residues to assess the chain-length specificity of PPK1 (11). Poly P longer than 23 P_i residues were processed by PPK1 completely in 1 h, whereas Poly P of 10 residues or fewer required more than 6 h of incubation. Combination of these methods is essential to measure both long- and short-chain Poly P.

Metachromatic Staining

Toluidine blue binding shows a broad chain-length specificity but is relatively insensitive. This method was used to measure poly P synthesizing activity in crude extracts of *Burkholderia cepacia* (12). Recently, a simple and fast method suitable for large-scale analysis of Poly P in various mutant strains of *Saccharomyces cerevisiae* has been described (13, 14).

^{31}P-NMR Spectroscopy

This physical method, applied to measure Poly P in intact cells of *Phycomyces blakesleeanus*, showed the effect of anoxia and respiratory chain inhibitors azide and cyanide on the Poly P content (15). The relative Poly P content was estimated as the intensity ratio of the "core Poly P" signal (signal assigned to central

Poly P residues) to the intracellular P_i signal (16). This analysis indicates the role of Poly P as energy and/or a phosphate reserve during sporangiospore development. A bioreactor coupled with ^{31}P NMR spectroscopy was used to analyze both intra- and extracellular phosphorylated metabolites including Poly P in *S. cerevisiae* (17).

Electron Ionization Mass Spectrometry

The high selectivity of electron ionization mass spectrometry (ESI-MS) allows the detection of different Poly P species without prior separation by ion chromatography or capillary electrophoresis (18). ESI-MS does not require the incorporation of UV-absorbing chromophores for detection of phosphates. Limits of detection ranged from approximately 1 to 10 ng/ml.

Cryoelectron Tomography and Spectroscopy Imaging

Characterization of intact subcellular bodies in whole bacteria was made possible by this combination of techniques and proved useful in recognizing P_i-rich bodies in *Deinococcus grandis* and *Caulobacter crescentus* (19). This method can also distinguish between carbon-rich and P_i-rich bodies. Cryoelectron microscopy and tomography allowed the study of the biogenesis and morphology of these bodies at resolution greater than 10 nm, whereas spectroscopic difference imaging provided a direct identification of their chemical composition.

Protein Affinity Labeling In Vivo

Visualization of Poly P at the ultrastructural level in yeast is achieved by a novel technique, which uses the affinity of a recombinant Poly P-binding domain (PPBD) of *E. coli* PPX1 (20). To directly demonstrate Poly P localization in *S. cerevisiae* on resin sections prepared by rapid freezing and freeze substitution, specimens were labeled with epitope-tagged PPBD, followed by the detection of the tag by an immunocytochemical procedure. By this method, localization of Poly P in intact cells at the electron microscopy level is studied with much higher specificity and resolution than with other methods.

CELLULAR LOCATION

Volutin Granules

These electron-dense storage particles of Poly P are more widespread than generally recognized. Seen as purple on histochemical staining with basic blue dyes, they are metachromatic. First observed over a century ago, the granules were most prominent in *Mycobacterium* sp. and in *Corynebacterium diphtheriae*. Finding the granules in *Corynebacterium glutamicum* (21), a nonpathogen, shows that as in *Mycobacterium*, they are not diagnostic of a disease state. *Vibrio cholerae* (01, biotype El Tor) accumulates Poly P principally as large granules (22).

The alga *Chlamydomonas reinhardtii* contains cytoplasmic vacuoles often filled with a dense granule that is released from the cell by exocytosis (23); purified granules contain Poly P complexed with Ca^{2+} and Mg^{2+} as the predominant inorganic components. Immunoelectron microscopy demonstrates a 70-kDa protein localized in vacuolar granules and the trans-Golgi network in a cell-wall-deficient mutant (23).

Chemical and immunological analysis revealed the presence of a calcium-Poly P complex enveloped by an amphiphilic, solvating polyester, poly-(R)-3-hydroxybutyrate (24). In the outer membranes of *Haemophilus influenzae* and *E. coli*, the complex enables the uptake of DNA plasmids.

Acidocalcisomes

These Ca^{2+}-Poly P-rich vacuoles are responsible for the flux of Ca^{2+} into the cytosol. The acidocalcisome has been described in the trypanosomatids (25–31) as electron-dense particles in the parasites as seen by transmission electron microscopy. Mass-dense granules of *D. discoideum* are homologous to the

acidocalcisomes described in protozoan parasites and are linked to the function of the contractile vacuole (32).

All the known acidocalcisomes in situ or purified contain large amounts of Poly P, pyrophosphate (PP_i), divalent metal ions, and the enzyme DdPPK2 (see POLY P SYNTHESIS IN EUKARYOTES, below). They are acidic, calcium-storage compartments with proton pumps located in their membranes that have been described in several unicellular eukaryotes, including trypanosomatid and apicomplexan parasites, algae, and slime molds.

Acidocalcisomes have also been found in bacteria, as in *Agrobacterium tumefaciens* and in *Rhodospirillum rubrum* (32). The number of acidocalcisomes and the amount of PP_i and Poly P in *R. rubrum* increases when the bacteria are grown in light. Human platelet-dense granules contain Poly P and are similar to acidocalcisomes of bacteria and unicellular eukaryotes (33). Thus, acidocalcisomes with volutin granules of Poly P bodies have been conserved during evolution from bacteria to humans (34).

Other Vacuoles, Nuclei, and Mitochondria

The vacuoles of *Neurospora crassa*, grown in minimal medium, contain a 1:1 ratio of basic amino acids and Poly P (35). The Poly P of the *Neurospora* vacuole are mainly in the range of 15 to 45 residues in chain length (36) and are associated with polycationic spermidine and Mg^{2+}. Despite the large amounts of basic amino acids in vacuoles, they remain iso-osmotic with the cytosol in part because of the interaction between polyanionic Poly P and the basic amino acids. Poly P also helps to relieve the osmotic burden, allowing the vacuoles to remain small during cytoplasmic streaming.

Purified fractions of cytosol, vacuoles, nuclei, and mitochondria of *S. cerevisiae* possess Poly P with chain lengths characteristic of each compartment (37). Most (80% to 90%) of the total Poly P was found in the cytosol fractions, in contradiction to earlier observations. It had been presumed that vacuolar Poly P in yeast accounted for about 98% of the total, because mutants of vacuolar ATPase contained less than 2% Poly P (38). Recent studies showed that the vacuoles contained approximately 15% of the total cellular Poly P (39).

Isolated mitochondria of *S. cerevisiae* grown on glucose also possess acid-soluble, short-chain Poly P (<15 P_i residues), the levels of which depend on the P_i concentration in the growth medium (40). Mitochondrial fractions obtained by differential centrifugation on sucrose gradients of *D. discoideum* cell lysates also contained short-chain Poly P (~14 P_i) (N.N. Rao, M.R. Gómez-García, A. Kornberg, unpublished results).

Poly P has also been found in all higher eukaryotic organisms tested, where it is present in specialized tissues (e.g., liver, kidney, lungs, brain, and heart) and localized in various subcellular compartments, including mitochondria (41).

A large ion channel, isolated from rat liver mitochondria by water-free chloroform extraction, is remarkably similar to the polyhydroxybutyrate complex with calcium Poly P in the membranes of *E. coli* competent for the uptake of DNA. The behavior of the isolated mitochondrial channel resembles that of the ion-conducting module of the permeability transition pore channel (42).

Infrastructure in Bacteria: Cytoskeleton, Compartments, Nucleoid

The organization of the bacterial cell is a crucial element in the function of biosynthetic and metabolic pathways. Yet, in the traditional efforts to resolve and reconstitute a biological event in a bacterial cell, infrastructure had not been considered. Now, it has been documented that bacteria have filamentous and microtubular cytoskeletal networks as well as many discrete microcompartments (43).

Put simply, MreB and ParM both engage in the formation of actin-like filaments that are indispensable in the maintenance of cell

shape and the segregation of plasmids (44, 45). Reconstitution of the segregation of the R1 plasmid bound to the ends of Par M filaments has been observed (46). The ring that constricts the cell during division is made of FtsZ, which resembles tubulin in its structure and dynamic assembly (47).

Bacterial microcompartments, unlike the lipid vesicular organelles of eukaryotes, are formed by proteins. The first recognized was the carboxysome, which is involved in fixing carbon dioxide in autotrophs and cyanobacteria. Some 5 to 10 different polypeptides form a polyhedral shell not unlike that of bacteriophages, 100–200 nm across with a volume a thousandfold greater than that of a ribosome.

Another compartment, responsible for degrading propanediol, is composed of at least 14 different polypeptides, including 4 enzymes and 7 different polypeptides that comprise the shell. Based on the signature polypeptide sequences of the shell deduced from sequenced genomes, many more compartments can be inferred with diverse enzymes and functions.

The remarkable acidocalcisomes, responsible for Ca^{2+} flux and rich in Poly P, are judged to be present in bacteria as well as prominent in organisms, including human blood platelets. Poly P also has a role in maintaining the extended shape of the nucleoid and the cell envelope (48). In PPK1-mutant cells, the nucleoid is compacted, and the cytoplasm is detached in many places from the cell poles and borders.

Bacterial Ion Channel and DNA Entry

The polyhydroxybutyrate/calcium/Poly P complex present in bacterial membranes made competent for DNA entry is also the cation-selective conductance channel. A channel of similar structure has proven to be the mitochondrial permeability transition pore. The PHB/Ca^{2+}/Poly P complex, first isolated from competent bacterial cells, has the high conductance and cation selectivity of the natural channel (49).

POLY P SYNTHESIS IN BACTERIA

Prebiotic Poly P

Even though life's origin must remain speculative, the subject of how Poly P might be part of the process deserves to be considered far more than it has been. Phosphate rock in the primitive earth under high pressures and desiccation was likely a rich source of phosphate, some as Poly P. The seas with phosphate highly diluted may have left some precipitated in sedimentary rock on which early forms of life could thrive. Recall that Poly P is abundant in volcanic exudates and deep-sea steam vents.

That phosphate predominates in all life forms still does not exclude a role for other anions at the earliest stages. At such a time, silica gels and polysilicates, as now seen in diatoms, may have had morphologies with self-replicating properties that participated with Poly P in primitive cellular forms (50). Yet, indisputably being a phosphate world, remarkably little mention is made of Poly P in its prebiotic stages. Surely, the unique chemical and physical properties of Poly P and its ubiquity must have played key roles in the origin and survival of species.

Poly P Kinase: Principal Source of Poly P in Bacteria

PPK1, the highly conserved and most studied of the enzymes in Poly P metabolism, catalyzes the transfer of the terminal phosphate of ATP to an active-site histidine residue, the initial step in the processive synthesis of a long Poly P chain. The reaction is reversible but favors synthesis.

A recent listing of species possessing sequences homologous to *ppk1* includes 100 or more, among them some of the major bacterial pathogens (**Table 1**). PPK1 has also been found in a eukaryote, the slime mold *Dictyostelium discoideum* (see below). Most bacterial species have both PPK1 and PPK2, an enzyme that favors synthesis from Poly P of the nucleoside triphosphate, particularly GTP from GDP.

Table 1 Polyphosphate kinase 1 (PPK1) and PPK2 homologs among microorganisms

PPK1	PPK2	PPK1 and PPK2
Acidovorax avenae subsp. *citrulli*	*Bacillus thuringiensis*	*Aeromonas hydrophila*
Acinetobacter baumannii	*Bordetella bronchiseptica*	*Agrobacterium tumefaciens*
Bacillus halodurans	*Bordetella parapertussis*	*Arthrobacter aurescens*
Bordetella pertussis Tohama	*Brevibacterium linens*	*Bacillus anthracis*
Brucella suis	*Chromobacterium violaceum*	*Bacillus cereus*
Candidatus accumulibacter	*Congregibacter litoralis*	*Bacteroides fragilis*
Clostridium acetobutylicum	*Corynebacterium diphtheriae*	*Bradyrhizobium japonicum*
Enterobacter sp.	*Corynebacterium efficiens*	*Brucella melitensis*
Erwinia carotovora	*Corynebacterium glutamicum*	*Burkholderia cenocepacia*
Escherichia coli	*Corynebacterium jeikeium*	*Burkholderia mallei*
Halorhodospira halophila	*Dinoroseobacter shibae*	*Burkholderia pseudomallei*
Helicobacter acinonychis	*Erythrobacter litoralis*	*Burkholderia thailandensis*
Helicobacter pylori	*Francisella tularensis* subsp. *tularensis*	*Burkholderia vietnamiensis*
Klebsiella pneumoniae subsp. *pneumoniae*	*Francisella tularensis* subsp. *novicida*	*Burkholderia xenovorans*
Lactobacillus delbrueckii	*Francisella tularensis* subsp. *holarctica*	*Campylobacter fetus* subsp. *fetus*
Lactobacillus plantarum	*Kineococcus radiotolerans*	*Campylobacter jejuni*
Legionella pneumophila subsp. *pneumophila*	*Magnetococcus* sp.	*Caulobacter crescentus*
Legionella pneumophila subsp. Lens	*Methylococcus capsulatus*	*Chlorobium tepidum*
Leuconostoc mesenteroides	*Mycobacterium ulcerans*	*Deinococcus radiodurans*
Methylobacillus flagellatus	*Neisseria gonorrhoeae*	*Dichloromonas aromatica*
Methylococcus capsulatus	*Nitrospira multiformis*	*Frankia alni*
Mycobacterium leprae	*Oceanospirillum* sp.	*Geobacter metallireducens*
Neisseria meningitidis	*Planctomyces maris*	*Helicobacter hepaticus*
Nitrosomonas eutropha	*Plectonema boryanum*	*Jannaschia* sp.
Photorhabdus luminescens	*Propionibacterium acnes*	*Lactobacillus brevis*
Porphyromonas gingivalis	*Psychrobacter cryohalolentis*	*Lactobacillus casei*
Rhodofera ferrireducens	*Rhizobium leguminosarum*	*Magnetospirillum magneticum*
Salmonella enterica subsp. *enterica*	*Roseovarius* sp.	*Magnetospirillum magnetotacticum*
Salmonella typhi	*Silicibacter pomeroyi*	*Methanosarcina acetivorans*
Salmonella enterica subsp. *typhimurium*	*Sinorhizobium medicae*	*Methanosarcina mazei*
Serratia marcescens	*Staphylococcus aureus*	*Mycobacterium avium*
Serratia proteamaculans		*Mycobacterium gilvum*
Shewanella amazonensis		*Mycobacterium smegmatis*
Shewanella baltica		*Mycobacterium tuberculosis*
Shewanella loihica		*Mycobacterium vanbaalenii*
Shigella boydii		*Myxococcus xanthus*
Shigella dysenteriae		*Nostoc punctiforme*
Shigella flexneri 2a		*Paracoccus denitrificans*
Shigella sonnei		*Photobacterium profundum*
Sodalis glossinidius		*Prochlorococcus marinus*
Staphylococcus saprophyticus		*Pseudomonas aeruginosa*
Thermus thermophilus		*Pseudomonas entomophila*
Thiobacillus denitrificans		*Pseudomonas fluorescens*
Yersinia bercovieri		*Pseudomonas putida*
Yersinia enterocolitica		*Psychrobacter articus*
Yersinia frederiksenii		*Ralstonia eutropha*
Yersinia intermedia		*Ralstonia metallidurans*
Yersinia mollaretti		*Ralstonia solanacearum*

(*Continued*)

Table 1 (*Continued*)

PPK1	PPK2	PPK1 and PPK2
Yersinia pestis		*Rhizobium etli*
Yersinia pseudotuberculosis		*Rhodobacter sphaeroides*
		Rhodopseudomonas palustris
		Rhodospirillum rubrum
		Roseobacter denitrificans
		Sinorhizobium meliloti
		Staphylococcus epidermidis
		Staphylococcus haemolyticus
		Streptomyces avermitilis
		Streptomyces coelicolor
		Synechococcus elongatus
		Synechococcus sp.
		Vibrio alginolyticus
		Vibrio cholerae
		Vibrio harveyi
		Vibrio parahaemolyticus
		Vibrio vulnificus
		Xanthomonas axonopodis
		Xanthomonas campestris

Mycobacterium tuberculosis

Among the many bacterial pathogens that depend on PPK1 and Poly P for their virulence as well as other functions, *M. tuberculosis* deserves special mention. This bacterium is remarkable for its long growth cycle, distinctive and visible accumulations of Poly P, and the current concern on how to cope with the global terror of a drug-resistant, deadly disease.

Tuberculosis is an ancient disease and a latent infection that is present in much of the world's population, especially the impoverished third world. The disease has emerged in patients with impaired immunity such as those infected with human immunodeficiency virus-acquired immunodeficiency syndrome (HIV-AIDS) and those undergoing cancer chemotherapy. Not only is there widespread resistance to available drugs to treat the disease, but also the prospect of new drugs seems unlikely because of the long and costly process of drug discovery and development by the pharmaceutical industry.

As noted above (in CELLULAR LOCATION), Poly P and *M. tuberculosis* have been connected for a long time. Particles that stain metachromatically appear purple with a dye, such as toluidine blue, were called volutin and observed prominently in bacteria, such as *Mycobacterium* sp. and *Corynebacterium* sp. (51). A half century later in 1946, these particles were identified as Poly P (52). This discovery came at a time when the mitochondrial source of ATP was still unknown. The possibility that Poly P might be a key intermediate in the energetics of aerobic metabolism flickered and was then extinguished with the emergence of ATP synthetase.

On the positive side of the current picture of tuberculosis are new insights about the growth and metabolic patterns of the bacterium and the structural features of PPK1 as a target for rational drug design. Recent studies (53) have shown the need for PPK1 and Poly P in the unusually long growth cycle and dormancy of the organism. X-ray diffraction data of the unique ATP-binding site, highly conserved in PPK1, reveal a target not found in any host cell.

PPK1: Structural Studies

Among the growing list of enzymes that make and hydrolyze Poly P, PPK1, is the most

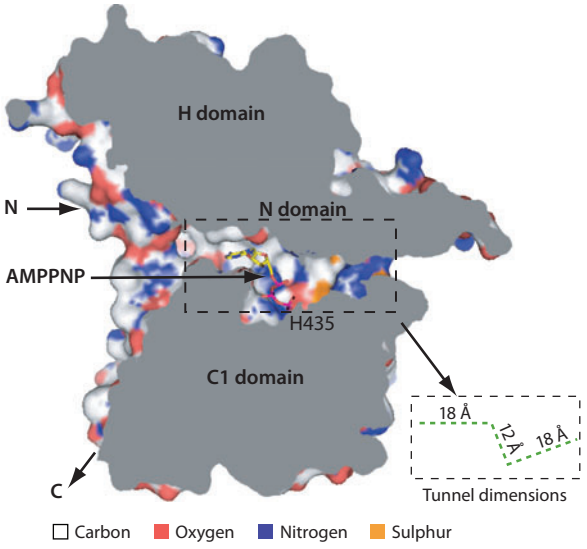

Figure 2

Cutaway illustration of the solvent-accessible surface of PPK1 structure. The front portion of the protein has been cut away to show the tunnel, which contains the AMPPNP (β-γ-imidoadenosine 5-phosphate) molecule. The N and C termini are indicated by arrows. Autophosphorylation site H435 is labeled in its position. The surface is colored by element as defined in the key. The dimension of the tunnel is indicated in the box with dashed lines.

widely conserved (54, 55). To reveal the catalytic mechanism of PPK1 and to investigate the structure of the enzyme-AMPPNP (β-γ-imidoadenosine 5-phosphate)-inhibitor complex, the full length *E. coli* PPK1 (EcPPK1) was crystallized (56). PPK1 forms an interlocked dimer from 80-kDa monomers containing four structural domains. The active site is located in a highly conserved structural tunnel, with a unique ATP-binding pocket, and probably accommodates the translocation of newly synthesized Poly P (56). The first step of Poly P synthesis is the autophosphorylation of the enzyme protein. Previous studies indicated that two histidine residues, H435 and H454, were the probable autophosphorylation sites (57). However, recent studies indicated only H435, which functions as a nucleophile attacking the phosphodiester bond of the γ-phosphate group of ATP (**Figure 2**).

PPK1 homologs have been found in over 100 prokaryotic species, including 20 major pathogens, and, to date, only in a few eukaryotes, including the DdPPK1 of *D. discoideum* and EcPPK1-like enzyme in *Candida humicola* (58–60). The deduced amino acid sequence of DdPPK1 shares 30% identity and 51% similarity with EcPPK1. DdPPK1 contains 1050 amino acid residues compared to only 688 in EcPPK1; the additional 370–amino acid N-terminal domain, occupying one-third of DdPPK1 in length, shows no homology to either EcPPK1 or any other protein in the GenBank database. Among the 11 conserved residues essential for a presumed Poly P tunnel, 10 are present in DdPPK1; 14 out of 16 residues in the ATP-binding pocket are also located in DdPPK1 (**Figure 3**). The two histidine residues (H435, critical for autophosphorylation, and the other, H592, required as a general acid catalyst in PPK1) and four other highly conserved residues (corresponding to D470, E623, S610, and T458 in EcPPK1) are also present in DdPPK1. Thus, DdPPK1 may share similar structural features with prokaryotic enzymes in the unique ATP-binding pocket and Poly P tunnel.

Poly P Kinase 2

Pseudomonas aeruginosa PAO1 mutants lacking *ppk1* are deficient in motility, quorum sensing, biofilm formation, and virulence in mouse models (61–63). Despite the lack of PPK1, the principal enzyme of Poly P synthesis, mutants still possess as much as 20% of the wild-type (WT) levels of Poly P (61), indicating another polyphosphate kinase, PPK2. Upon isolation, PPK2 is a 44-kDa protein with a predicted isoelectric point of 9.85. In the presence of Poly P_{15}, PPK2 migrates as a unit of 385 kDa, probably an octamer (**Table 2**), and, in the absence of Poly P, is unstable and forms tetramers (64).

PPK2 activity from *P. aeruginosa* differs from PPK1 in two major features (**Table 2**). One is that PPK2 utilizes Poly P to make GTP (Poly P-driven nucleoside diphosphate kinase activity, PNDK) at a rate 75-fold greater than that of Poly P synthesis from GTP (54). The other feature is the relative selectivity for guanosine and adenosine nucleotides as donors and acceptors:

PPK2 uses GTP and ATP equally well in Poly P synthesis, but PPK1 is strictly specific for ATP. In the utilization of Poly P as a donor to nucleoside diphosphates, PPK2 uses GDP or ADP, whereas PPK1 has a >30-fold preference for ADP over GDP. Thus, PPK2, at least in isolated form, seems to be designed for synthesis of GTP from Poly P in contrast to PPK1, which strongly favors synthesis of Poly P and exclusively from ATP. Mn^{2+} is preferred over Mg^{2+} by PPK2 for Poly P synthesis activity, whereas the reverse is true for PPK1. The optimal level of Mn^{2+} for PPK2 is reached at 10 mM, but levels of even 25 mM are tolerated. Whether Mn^{2+} serves by binding the nucleoside triphosphate or at the active site of the enzyme, or both, is still unknown. A requirement for Mn^{2+} is shared by many enzymes, including the nucleoside diphosphate kinase activity (NDK) of pyruvate kinase, which is sustained by Mn^{2+} at the very low level of 0.05 mM (54, 64).

PPK2 in *P. aeruginosa* is induced >100-fold from barely detectable levels when the culture approaches stationary phase. This is the very stage when GTP is needed for the synthesis of alginate (65, 66), the exopolysaccharide that envelops the bacterium, and possibly for other functions of GTP. Thus, the predominant activity of isolated PPK2, which is to use Poly P for the generation of GTP, is reflected in its appearance just when needed in cells in culture. By contrast with PPK2, the cellular levels of the classic ATP-driven NDK remain near constant in log and stationary phases of *P. aeruginosa*.

In contrast to *P. aeruginosa*, which contains both PPK1 and PPK2, *C. glutamicum*, a gram-positive bacterium, has only the PPK2 group of enzymes. The genes *ppk2A* and *ppk2B* encode proteins that share 57% and 64% sequence identity with *P. aeruginosa* PPK2 and 51% sequence identity with each other (67). Unlike PPK2 from *P. aeruginosa*, the enzyme PPK2B has a preference for Poly P synthesis from ATP or GTP over the reverse NAD kinase reaction. This enzyme is responsible for the accumulation of high concentrations (600 mM) of both soluble and granular Poly P.

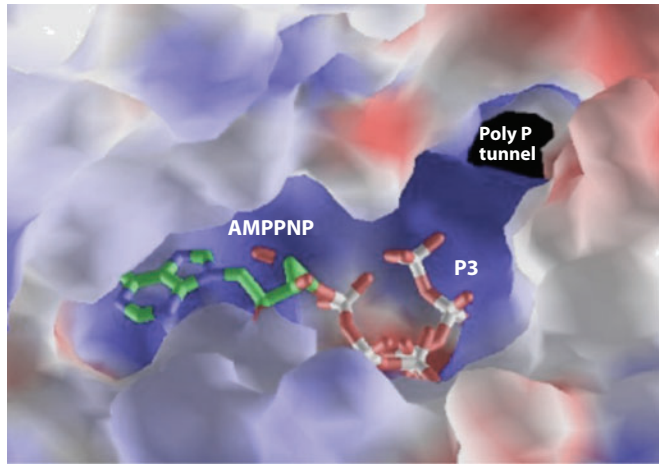

Figure 3

The active site of *E. coli* Poly P kinase 1, including bound substrate, in this case AMPPNP, and the putative inorganic polyphosphate (Poly P) tunnel. P3 indicates the growing chain of Poly P. The data are kindly provided by Dr. Wenqing Xu at the University of Washington, Seattle, and Dr. Sam Lee then at ICOS Corporation, Bothell, Washington.

Table 2 *P. aeruginosa* polyphosphate kinase 1 (PPK1) and PPK2 in the synthesis and utilization of polyphosphate (Poly P)

	PPK1[a]	PPK2
Synthesis (NTP → Poly P)		
ATP or GTP	ATP only	ATP or GTP
Metal ion	Mg^{2+}	Mn^{2+}
Poly P stimulation	None	Yes
V_{max} (units/mg protein)	2.1×10^6	6.7×10^6
Poly P product residues	500–800	200–800
Processivity	Yes	Yes
Utilization (Poly P → NTP)		
ADP/GDP	34	0.9
Poly P size preference	Long	Short
Metal ion	Mg^{2+}	Mg^2
V_{max} (units/mg protein)	5.1×10^5	5.0×10^8
Processivity	Yes	Yes
Synthesis/utilization		
V_{max} ratio	4.1	0.013
Size		
Subunit size	80 kDa	44 kDa
Multimeric size	320 (tetramer)	385 (octamer)
Growth		
Stage of induction		
Stationary/log	<1	>100

[a]His-tagged *P. aeruginosa* PPK1 was a gift from S. Lee (ICOS Corporation).

Table 3 Polyphosphate kinase 1 (PPK1) and PPK2 homologs[a] among bacterial pathogens

PPK1	PPK2	PPK1 and PPK2
Bordetella pertussis	*Bordetella bronchiseptica*	*Bacillus anthracis*
Helicobacter pylori	*Bordetella parapertussis*	*Brucella melitensis*
Legionella pneumophila	*Bacillus thuringiensis*	*Mycobacterium tuberculosis*
Mycobacterium leprae	*Corynebacterium diphtheriae*	*Pseudomonas aeruginosa*
Neisseria meningitidis	*Francisella tularensis*	*Staphylococcus haemolyticus*
Salmonella enterica spp. *typhi*	*Neisseria gonorrhoeae*	*Vibrio cholerae*
Shigella dysenteriae	*Staphylococcus aureus*	
Yersinia pestis		

[a]Based on a Basic Local Alignment Search Tool (BLAST) search with *E. coli* PPK1 and *P. aeruginosa* PPK2 of the NCBI Microbial Protein/Genome database for homologs with an E value $<1 \times 10^{-10}$.

PPK2: Widely Conserved Sequence

In the *P. aeruginosa* genome, two PPK2 sequence homologs are present: homolog 1 (NP_251118) and homolog 2 (NP_252145). Biochemical analysis of the two PPK2 homologs defined homolog 1 as another PNDK with 41% identity with PPK2; homolog 2 has significant sequence similarity to a Poly P:AMP phosphotransferase (PAP) from *Acinetobacter johnsonii* (67–70; H. Zhang, K. Ishige, unpublished data).

Among other microorganisms, PPK2 homologs have been identified in over 100 species, including many of the major pathogens (**Table 3**). The importance of PPK1 in bacterial metabolism and virulence of pathogens (61, 71, 72) has been exploited as a target for the discovery of antibiotics that inhibit the enzyme (S. Lee, personal communication). Now with the recognition that PPK2 may contribute to the synthesis of alginate of, and the mucoidy associated with, virulence, PPK2 may like PPK1 prove to be a target for the discovery and design of novel antibiotics.

POLY P SYNTHESIS IN EUKARYOTES

DdPPK1: A Conserved Bacterial Enzyme in *D. discoideum*

PPK1, the principal enzyme responsible for reversible synthesis of Poly P from the terminal phosphate of ATP, is highly conserved in bacteria and archaea. *D. discoideum*, a social slime mold, is one of a few eukaryotes known to possess a PPK1 homolog (DdPPK1) (8, 73). Compared with PPK1 of *E. coli*, DdPPK1 contains the conserved residues for ATP binding and autophosphorylation but has an N-terminal extension of 370 amino acids lacking homology with any known protein. Poly P stimulates DdPPK1 activity in vitro and also promotes oligomerization of the enzyme. The Poly P products of DdPPK1 are heterogeneous in chain length and shorter than those of *E. coli*. The unique DdPPK1 N-terminal domain is necessary for its enzymatic activity, cellular localization, and physiological functions. Mutants of DdPPK1 are defective in development, sporulation, and predation, and in late stages of cytokinesis and cell division. Unlike WT cells, which are almost all mononucleated, about 30% of DdPPK1 mutant cells are multinucleated. WT cells completed cell division in a few minutes after anaphase, whereas nearly half of the mutant cells failed to divide (60). DdPPK1 is the first eukaryotic PPK1 to be studied, but it seems likely that more will be found. The yeast *C. humicola* has a PPK1 activity and a cloned DNA fragment with 34% homology to the deduced protein sequence in *E. coli* PPK1 (59).

DdPPK2: An Enzyme That Forms an Actin-Like Filament Concurrently with the Synthesis of Poly P

Mutants of *D. discoideum* lacking DdPPK1 (58), the highly conserved bacterial enzyme, still possess significant PPK activity. This activity (that

of DdPPK2) is associated with a vacuole, the acidocalcisome, that contains large amounts of Poly P and is responsible for Ca^{2+} flux in cells. The vacuole is also prominent in *Trypanosoma cruzi*, *Leishmania major*, *Toxoplasma gondii*, and the alga *Chlamydomonas*.

When the DdPPK2 activity was purified, it proved to be a tetramer of three actin-related proteins (Arps): Arp1 is known as the major subunit in the dynactin complex that promotes movement of membrane vesicles, Arp2 is known as part of a complex that plays a role in cell motility, and Arp28 was previously unknown. In the presence of ATP, the globular Arp tetramer polymerizes into an actin-like filament concurrent step by step with the synthesis of a long Poly P chain. The actin and Poly P form a complex that is susceptible to PPN1 but not exopolyPase. The localization and the physiologic functions of the actin-Poly P complex have not been determined (5).

Recent studies have established that prokaryotes also possess and depend on skeletons of microfilaments that resemble actin and tubulin (43, 44, 47). These features determine cell shape and also are likely involved in a variety of cellular functions, some of which might depend on Poly P. Of special interest is that, in the synthesis of Poly P by PPK1 of *P. aeruginosa*, filamentous assemblies are formed, and this Poly P synthesis is inhibited by phalloidin, as is DdPPK2, but with a different stoichiometry (M.R. Gómez-García, H. Hayashi, & A. Kornberg, unpublished data).

It is important to note that DdPPK2 was obtained from a *D. discoideum* membrane fraction that contains the acidocalcisomes. Poly P synthesis measured in these isolated vacuoles is inhibited 75% by 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a proton-motive-force (PMF) poison (M.R. Gómez-García & A. Kornberg, unpublished data). Thus, the actions of the vesicular PMF-driven synthesis of the phosphoanhydride bonds of Poly P resemble the synthesis of inorganic pyrophosphate (74) and ATP (75). The G-to-F transitions of DdPPK2 and Poly P may play a vital role in the contractility and other motor functions of the *D. discoideum* vacuole.

Other PPK Activities

The mechanisms for Poly P synthesis are largely described in bacteria, but little is known about them in eukaryotes except for a PPK1 homolog in the yeast *C. humicola* G1 (59) and in the soil amoeba *D. discoideum*. The amino acid similarity in *C. humicola* G1 with *E. coli* is 32% and with *Helicobacter pylori* J99 is 34%. The DdPPK1 of *D. discoideum* also is the main source of its Poly P. DdPPK1 contains the conserved residues for ATP binding and autophosphorylation in EcPPK1, but has an N-terminal extension of 370 amino acids, lacking homology with any known protein, and is essential for both biologic and catalytic activities (60).

In *S. cerevisiae*, transcriptional analysis has failed to detect a *ppk* gene, nor has a robust in vitro Poly P–synthesizing activity been observed (8, 22), despite the intracellular abundance of Poly P (76, 77). Using whole-genome DNA microarray analysis, under conditions of P_i starvation, four genes (*PHM1*, *PHM2*, *PHM3*, and *PHM4*, also named *vtc2*, *vtc3*, *vtc4*, and *vtc1*, respectively) were demonstrated to play a role in metabolism of Poly P. These genes are also involved in a vacuolar transporter chaperon (78) and function as regulators of vacuolar H^+-ATPase activity (22, 78, 79). Mutations result in severely restricted Poly P accumulation under conditions of "polyphosphate overplus," i.e., incubation in high P_i media, following a period of P_i starvation (22, 80). The Δ*vtc3* mutant has Poly P at a level of 40% of the WT but of a smaller median size. Poly P was undetectable in *vtc4* and *vtc1* mutants and in the *vtc2vtc3* double mutant, indicative of their involvement in the accumulation of Poly P; *vtc4* is also required in the fusion of P_i-containing vesicles with the vacuolar membrane (81) and the consequent accumulation of Poly P in the vacuole. Yet, the enzyme activities of these gene products are still unclear with regard to Poly P synthesis.

Table 4 Polyphosphate kinase 1 (PPK1) and PPK2 homologs[a] among Eukaryota

PPK1	PPK2
Aedes aegypti	*Aedes aegypti*
Anopheles gambiae	*Anopheles gambiae*
Caenorhabditis remanei	*Bombyx mori*
Candida humicola	*Drosophila simulans*
Dictyostelium discoideum	*Plasmodium yoelii yoelii*
Plasmodium yoelii yoelii	
Strongylocentrotus purpuratus	

[a]Based on a Basic Local Alignment Search Tool (BLAST) search with *E. coli* PPK1 and *P. aeruginosa* PPK2 of the NCBI Eukaryota Protein/Genome database for homologs with an E value $<1 \times 10^{-10}$.

Expanded genome databases and improved search engines have disclosed the most remarkable conservation of the bacterial PPK1 and PPK2 sequences among a wide variety of eukaryotic species in addition to DdPPK1 (**Table 4**). Clearly, studies are needed to identify the enzymes encoded by these sequences and perhaps provide applications of medical or industrial value.

Proton Motive Force as a Source of Poly P

Our approach to finding the multiple functions of Poly P has been to discover and characterize the enzymes that make and use it. Invariably, the source of Poly P in these reactions is ATP except in a single instance in which GTP can substitute. The absence of such an enzyme in an organism, and the lack of a sequence homologous to encode it, has raised the possibility that Poly P can be made directly by a PMF bypassing ATP as an intermediate. Recall that prebiotically, Poly P was abundant on earth long before biologic molecules. By virtue of its capacity as a potent phosphorylating agent, Poly P is a plausible source of energy and phosphate in the early evolution of our phosphate world. As a potent chelator of metal ions, the variety of reactions that exploit Mg, Mn, Ca, Fe, and Co facilitate a multitude of reactions, including the maintenance of ATP levels in a cell. As a metabolic regulator, Poly P has a unique role in the homeostasis of P_i, metals, and essential nutrients. The possibility of PMF as a major source of Poly P needs to be studied in a defined, vesicular system in which PMF can be generated and regulated. (The acidocalcisome has a potent extractable synthetic enzyme, DdPPK2, which uses ATP to make Poly P.) Studies of the intact vesicle may provide the means to observe that ATP can be bypassed and that PMF is used directly in the synthesis of Poly P.

UTILIZATION OF POLY P

EndopolyPase: Essential in Yeast

PPN1 cleaves long chains of Poly P distributively to generate shorter chains: a partial digest of 60 residues (Poly P_{60}) and tri Poly P (P_3) (82) and a limit digest of P_i and P_3 (83). The enzyme is widely distributed in animal cells and is most abundant in yeast, from which it was purified and shown to be a homotetramer of a 35-kDa subunit (82, 83); the encoding yeast gene is *PHM5*. Subsequent studies showed that the yeast enzyme is derived from a 78-kDa polypeptide (674 amino acids) of vacuolar origin, which is activated by a protease to generate the 35-kDa subunit (352 amino acids). The protease-processed PPN1 has been purified to homogeneity, and the protease cleavage sites determined. Both termini of prePPN1 and the posttranslational modification of N-glycosylations are essential for the protease-activated maturation of PPN1. The PPN1 gene has been cloned, sequenced, overexpressed, and deleted. Null mutants accumulate long-chain Poly P and are defective in growth in minimal media. A double mutant of PPN1 and scPPX1 (a potent exopolyPase) loses viability rapidly in a stationary phase. Whether this phenotype is a result of the excess of long-chain Poly P or the lack of the shorter chains is still unknown (84). A high-precision, multigenome-scale annotation (85) has identified a human gene associated with melanomas as *PPN1*. Possibly relevant are the observations that mTOR kinase, a key step in the proliferation of mammary cancer cells, is activated by Poly P and that the introduction of a yeast exopolyPase gene into the cancer cells

interrupts mTOR action and cell growth. Inasmuch as additional analytic reagents are needed for Poly P studies, overexpression of the processed form of PPN1 would be of great value, particularly in instances when the chain termini of Poly P are blocked and unavailable for analysis by the actions of exopolyPases and PPKs.

Poly P:AMP Phosphotransferase

The PAP enzyme enables Poly P to be used in place of ATP to elevate AMP to the triphosphate level. The PAP reaction begins

$$\text{AMP} + \text{Poly P}_n \rightarrow \text{ADP} + \text{Poly P}_{n-1},$$

followed by PPK1, where

$$\text{ADP} + \text{Poly P}_{n-1} \rightarrow \text{ATP} + \text{Poly P}_{n-2}.$$

As an alternative to PPK1, action of the widely distributed AMP kinase restores AMP for another round of PAP:

$$2\text{ADP} \rightleftharpoons \text{AMP} + \text{ATP}.$$

The PAP enzyme was first purified from *Acinetobacter* (86) and was shown to be processive with highest activities with Poly P lengths in the range of 20 to 40 residues. PAP has also been identified in *P. aeruginosa* (87), *Myxococcus xanthus* (88), and *Bacillus cereus* (89) and can be used with AMP as an economically attractive reagent in place of the more costly ATP (90).

Structure and Function of Exopolyphosphatases

Exopolyphosphatases (exopolyPases, PPXs) hydrolyze and processively release the terminal phosphate from linear Poly P containing three or more phosphoanhydride bonds. Based on the primary structure, they are classified into two types. The prototypic cytoplasmic PPX1 from *S. cerevisiae* (scPPX1) and PPX from fungi and protozoa belong to the superfamily of DHH phosphoesterases (named after the conserved Asp-His-His motif) (91). scPPX1 hydrolyzes linear Poly P chains longer than pyrophosphate and is evolutionarily related to the well-characterized family II pyrophosphatases, despite significant functional differences (92). *E. coli* contains two PPXs: PPX1 and GPPA (PPX2) present in other Eubacteria and Archaea and belong to another superfamily that includes sugar kinases, actin, heat shock protein hsp 70, and the prokaryotic cell cycle proteins (93–95). PPX1 of *E. coli*, an enzyme of 513 amino acid residues, with a molecular mass of 58.1 kDa, forms dimers in solution (93, 96). The P_i product is released in a processive manner from Poly P chains as long as 750 P_i residues. This enzyme requires both Mg^{2+} and KCl for maximal activity.

Limited proteolysis of PPX1 showed that it contained two functional domains: an N-terminal catalytic domain and a C-terminal domain responsible for Poly P binding and processivity (96). Recent studies of the crystal structure of PPX1 at 2.2-Å resolution revealed the presence of four domains (97). Domains I and II displayed structural similarity with one another and shared the ribonuclease H-like fold. Domain III showed structural similarity to this N-terminal, HD domain (named afer the conserved doublet of predicted catalytic residues, histidine and aspartic acid) of SpoT. The smallest domain, domain IV, has structural resemblance with cold-shock-associated RNA-binding proteins. The putative PPX active site is located at the interface between domain I and II, and the crystal structure revealed a dimeric enzyme with a deep canyon, the probable site of Poly P binding located at the dimer interface (**Figure 4**).

PPX/guanosine pentaphosphate phosphohydrolase (PPX2/GPPA) enzymes play important roles in the bacterial stringent response induced by starvation. A purified preparation of PPX2 from *E. coli* liberates P_i by processive hydrolysis of the phosphoanhydride bonds of Poly P chains (750 residues) or by hydrolysis of the 5′-γ-phosphate of guanosine 5′-triphosphate 3′-diphosphate (pppGpp) to guanosine 5′-diphosphate 3′-diphosphate (ppGpp or "magic spot"). A high-resolution crystal structure of the *Aquifex aeolicus* PPX2/GPPA protein

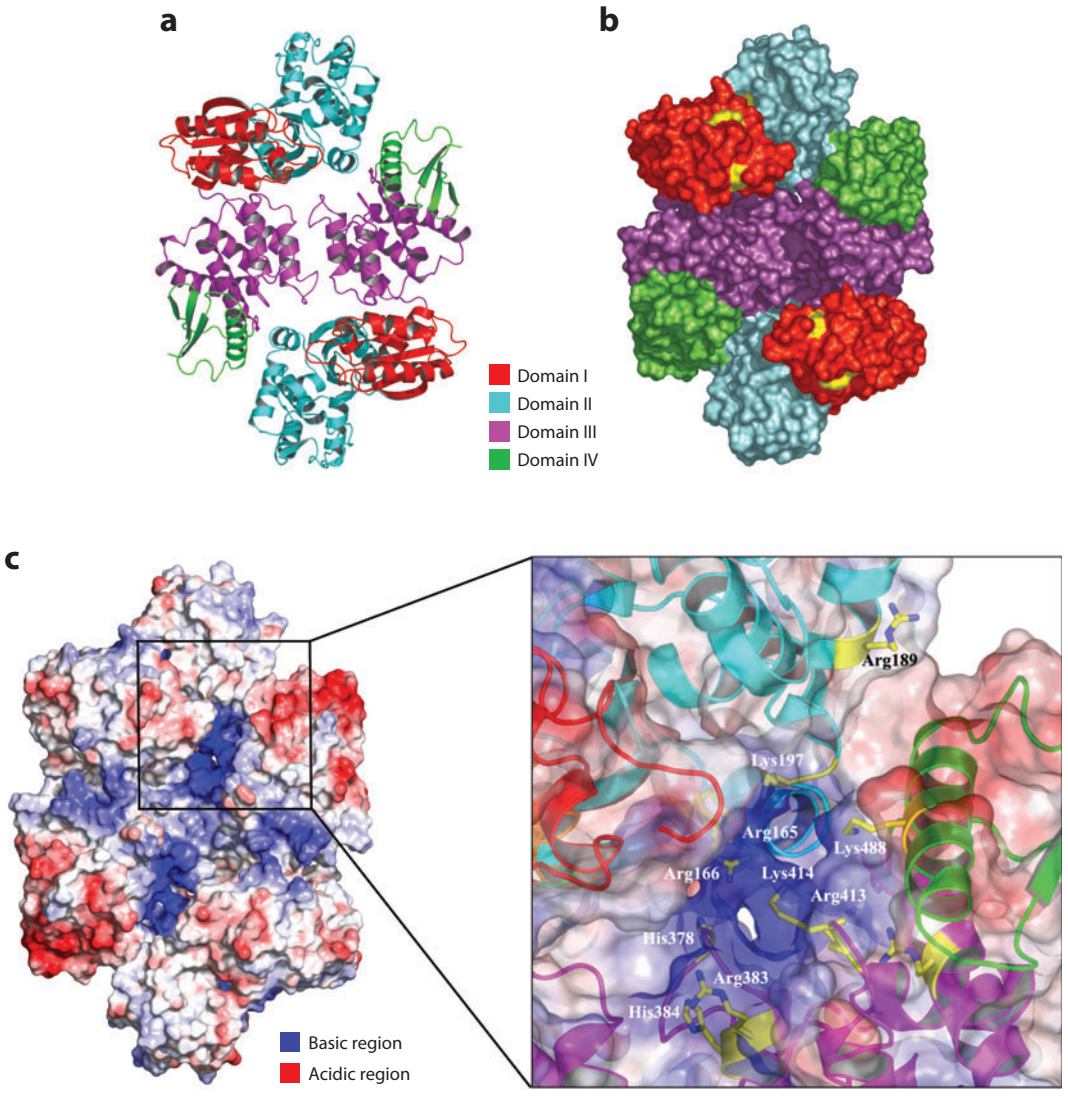

Figure 4

Structure of the *E. coli* PPX dimer and the putative active-site canyon. (*a*) Ribbon representation of the PPX dimer, (*b*) molecular surface representation showing the canyon that runs along the dimer interface, (*c*) electrostatic potential map, showing regions of positive potential within the putative Poly P binding cleft. A blow-up of the basic region of the cleft is shown, Rangarajan et al. (97).

revealed a two-domain structure with an active site located in the interdomain cleft. This provides the enzyme with a structural flexibility previously described as a "butterfly-like" cleft opening around the active site in other actin-like superfamily proteins. Ca^{2+} was observed at the center of the crystal, substantiating that PPX2/GPPA enzymes use metal ions for catalysis (98). A recent study wherein the structure of the PPX of *E. coli* was determined to 1.9-Å resolution by X-ray crystallography, shows the presence of an aqueduct that passes through the enzyme providing a physical basis for the enzyme's high processivity (99). This PPX is also

known as an ASKHA (acetate and sugar kinases, *hsp* 70, actin) phosphotransferase with an active site found in a cleft between the two N-terminal domains. Most of the 29 sulfate ions bound to the PPX dimer occupy sites where the Poly P chain likely binds (**Figure 5**).

NAD Kinase: Poly P or ATP as Donor

NADP synthesis from NAD by phosphorylation of the 2′ carbon of the adenylate moiety of NAD is crucial for many cellular processes of metabolism and biosynthesis. The NAD kinase uses either Poly P or ATP as donor in *M. tuberculosis*, *Micrococcus flavus*, and *Bacillus subtilis*. In all other NAD kinases tested, including those in *E. coli*, *S. cerevisiae*, and the plant *Arabidopsis*, ATP is the only donor.

Among the NAD kinases that can use Poly P, that of *M. tuberculosis* might be expected by virtue of the large volutin deposits of Poly P and the use of Poly P in PPK1 and PPK2. Yet, *B. subtilis* employs Poly P for its NAD kinase despite lacking both PPK1 and PPK2. Of considerable interest will be the binding sites of these several kinases and their phylogenetic relationships. A start in this direction is the analysis of the crystal structure of the *M. tuberculosis* kinase complexed with NAD observed at 2.7-Å resolution (100).

Glucokinase

An enzyme that employs Poly P as donor to form glucose 6-P from glucose has been found and purified from *M. tuberculosis* (101), *C. glutamicum*, *Propionibacterium shermanii* (102), and *Arthrobacter* sp. (103).

$$\text{Glucose} + \text{Poly P}_n \rightarrow \text{glucose 6} - \text{P} + \text{Poly P}_{n-1}.$$

In these organisms, ATP can also serve as donor, but generally with less favorable kinetics. An exception is *Microlunatus phosphovorus* (104), closely related to *M. tuberculosis*, but in this case strictly specific for Poly P as donor.

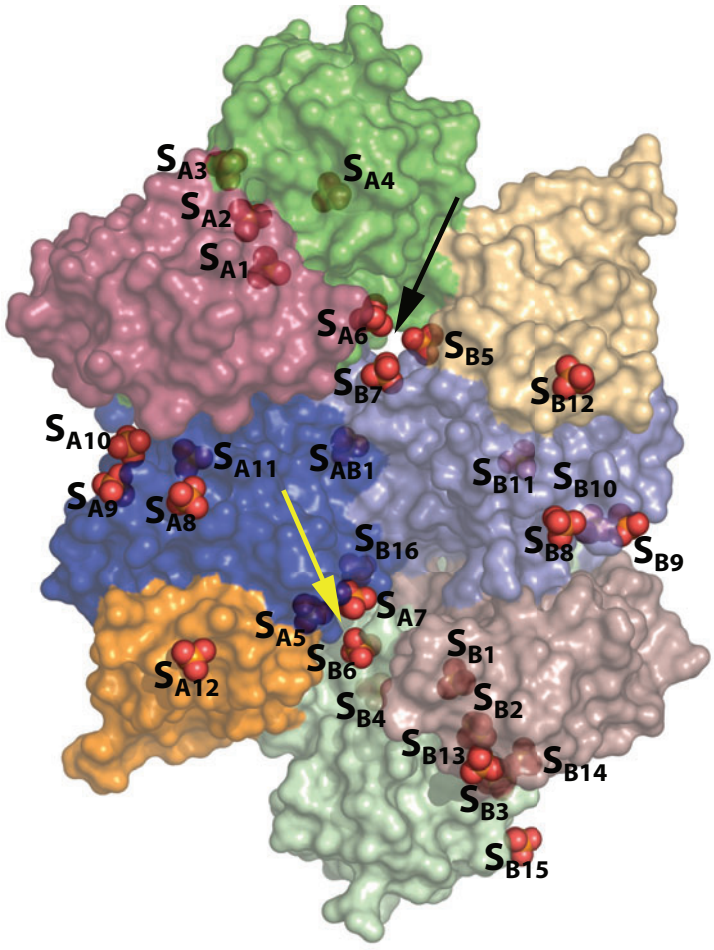

Figure 5

Surface representation of *E. coli* PPX1 dimer is shown with the 29 bound sulfate ions. To monomer A, shown in darker shades of color, 12 sulfates are bound (S_{A1}–S_{A12}), whereas in monomer B (in lighter shades of color), 16 sulfates are bound (S_{B1}–S_{B16}). A single sulfate ion is bound at a site of symmetry between the two monomers (S_{AB1}). The sulfate ions S_{B5}, S_{A6}, and S_{B7} and S_{A5}, S_{B6}, and S_{A7} are bound in the two aqueducts indicated by the arrows, Alvarado et al. (99).

TOR SIGNALING, CELL GROWTH, PROLIFERATION, AND APOPTOSIS

TOR (target of rapamycin) is a ser-thr protein kinase that is at the center of signaling pathways in cell growth and proliferation and is activated by autophosphorylation (105). TOR function is controlled by nutrient availability to ensure that protein synthesis is repressed when the supply of precursor amino acids is

insufficient. In animal cells, TOR integrates signals arising from the amino acid supply, cellular energy state, various hormones (such as insulin), and growth factors. TOR is conserved in all eukaryotes tested (106, 107). Poly P and PPK have multiple roles in cell growth and proliferation in some instances, interacting in association with TOR.

mTOR and the Proliferation of Mammary Cancer Cells

Unlike yeast and other lower eukaryotes, which contain two TOR genes, higher eukaryotes possess only one; mTOR (mammalian target of rapamycin) forms two functional protein complexes, mTORC1 and mTORC2. One of the key downstream targets of mTOR is PHAS-I (also called eIF4E-binding protein), which in its unphosphorylated state sequesters eIF4E, an mRNA cap-binding protein in the eIF4G elongation complex (106, 107). PHAS-I phosphorylation by mTOR releases eIF4E, stimulating initiation of both global translation and that of a particular set of proteins involved in cell growth and proliferation. This appears to be a crucial regulatory mechanism, inasmuch as overexpression of eIF4E alone can stimulate the transformation of rodent fibroblasts (108). The other major mTOR substrate is the kinase for the 40S ribosomal S6 protein (p70 ± S6 kinase), phosphorylation of which activates its enzymatic activity (109).

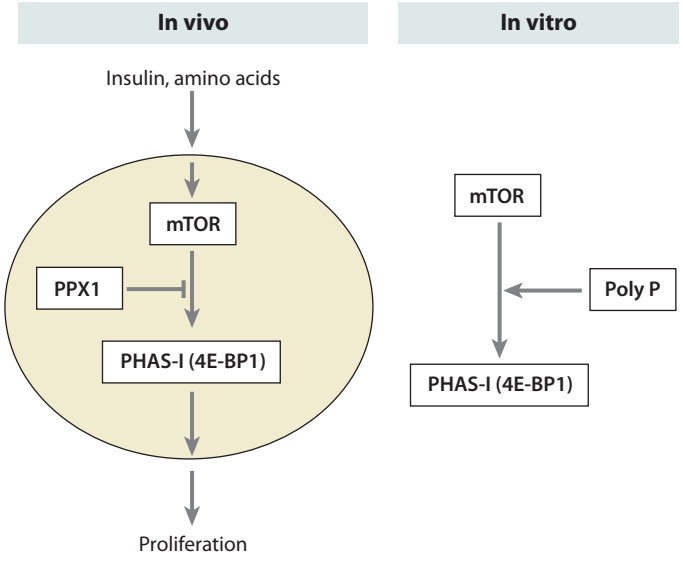

Figure 6

Scheme for a Poly P role in the mTOR signaing pathways. Poly P is proposed to act as a cofactor in the insulin and amino acid activation of mTOR.

Little is known about Poly P in animal cells beyond its widespread abundance in tissues and subcellular compartments, including the nucleus, mitochondria, membranes, acidocalcisomes, and vesicles (34). An exception is the role of Poly P in mTOR, which stems from attempts to reconstitute in vitro the activation of mTOR kinase activity observed in the cascade of kinase actions in the proliferation of mammary cancer cells (**Figure 6**). The extracellular signals for mTOR activation—insulin, amino acids, and mitogens—were not observed in cell extracts. A striking result was the ability of Poly P to stimulate manyfold the activation of mTOR, observed in its phosphorylation and in the phospho-mTOR activation of PHAS-I. The effect of Poly P depended on its chain length and concentration. This in vitro activation of mTOR phosphorylation was observed at concentrations of Poly P (0.15–1.5 μM) found in mammalian cells (41) and with Poly P chain lengths ranging from 15 to 750 phosphate residues. Subsequent mTOR autophosphorylation in vitro in the presence of Poly P and ATP induced a severalfold increase in mTOR kinase activity, similar to those previously reported after cells were treated with growth factors and nutrients (110). The ability of Poly P to activate mTOR in vitro was also seen in vivo with a human carcinoma cell line (MCF-7) genetically transfected to express yeast PPX1; the altered cells exhibited a greatly reduced response to mitogens, insulin, and amino acids as seen in their failure to activate mTOR and in the phosphorylation of its substrate, PHAS-I (**Figure 7**). In addition, the transfected cells were severely reduced in growth in a serum-free medium (111).

Consistent with the role of mTOR (TORC2 complex) in determining cell shape, PPX1-expressing cells were 25% smaller. Thus, the

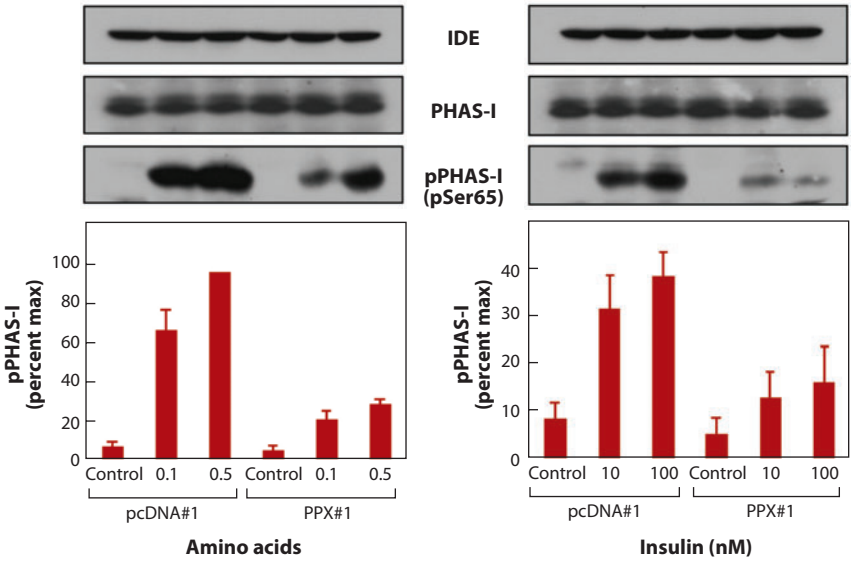

Figure 7

Polyphosphatase PPX1 reduces insulin and amino acid activation of mTOR activity in vivo. Serum- and amino acid–starved MCF-7 cells, either the pcDNA3.1 transfected (pcDNA#1) or PPX1-expressing (PPX1#1) cells, were incubated with either 0.1 and 0.5 of the normal levels of amino acids in MEM or 10 nM and 100 nM insulin. Cell lysates were analyzed by immunoblotting for an extraneous protein (IDE), total PHAS-I levels, or PHAS-I that was phosphorylated on Ser-65 (pSer65). (*upper*) Western blots of a representative experiment with arrows indicating the positions of IDE, total PHAS-I protein, and phosphorylated PHAS-I. The amount of pPHAS-I was quantitated, and the averages of three experiments are shown ± SE expressed as a percent of the maximally stimulated cells (111).

PPX1 effect appears to be pathway specific (111). On the basis of these findings, it appears that Poly P may serve as a regulatory factor in the activation of mTOR in the proliferative signaling pathways of animal cells. However, these studies need to be extended by determining Poly P levels in subcellular compartments of the transfected cells. Also, a control experiment in which a mutated yeast *ppx1* gene lacking the sequence for enzymatic activity would be relevant to the issue of Poly P regulation.

Although the focus in this section has been on mTOR, the presence of TOR activity is nearly universal from yeast to all animal cells. It can be presumed, from the actions of mTOR in the proliferation of cancer cells, that TOR activities have a variety of physiologic functions and that Poly P is a regulatory factor.

Modulation of Mitogenic Activity of Fibroblast Growth Factors by Poly P

The proliferation of normal human fibroblast cells can be enhanced by the addition of Poly P to culture media. The mitogenic activities of acidic fibroblast growth factor (FGF-1) and basic fibroblast growth factor (FGF-2) can also be enhanced by Poly P. A physical interaction between Poly P and FGF-2 was observed (112), and FGF-2 was both physically and functionally stabilized by Poly P. Furthermore, Poly P facilitated the FGF-2 binding to its cell surface receptors. The mechanism of growth stimulation by Poly P was similar to that reported with heparin. Heparin and heparin-like glycosaminoglycans are well-known potent modulators of FGF-1 and FGF-2, and they potentiate the mitogenic activity of both FGFs (113–117). Heparin sulfate stabilizes FGFs and binds to a site on the receptor and at least one site on

the growth factor. Several models propose an important role for heparin sulfate not only in facilitating FGF-2 binding to its receptor tyrosine kinase, but also in promoting signaling via the formation of receptor dimers. Such dimers are capable of *trans*-phosphorylation of the cytoplasmic domain of the receptor, leading to the generation of phosphotyrosine, an important initiator of the intracellular signaling pathway (114–117). Poly P may facilitate FGF-2 binding to its receptors and promote signaling through the same binding sites of heparin sulfate, FGF-2, and its receptors. Although Poly P and heparin are both polyanionic, they have different structures, and it is probable that the mechanism for modulation of FGF activity by Poly P is different from that of heparin. The level of growth stimulation by Poly P is higher than that by heparin (118). This also suggests that the binding sites between Poly P and FGFs could be different. Further analyses are needed to elucidate the detailed mechanism of interaction between Poly P and FGF-2.

Apoptosis

During apoptosis, nuclear chromatin undergoes condensation, and at this time, a calcium-dependent endonuclease (caspase-3) is activated and cleaves nuclear DNA at linker regions, creating DNA fragments of 180–200 bp in length. Addition of Poly P to human plasma cells (PCs) inhibited secretion of immunoglobulin and stimulated apoptosis. The Poly P stimulation was specific for PCs and myeloma (malignant PC) cell lines as well as for primary myeloma cells and B lymphoid cell lines; normal B cells, T cells, total blood mononuclear cells, and nonlymphoid cell lines were not affected. Poly P addition to the myeloma cell line activated caspase-3 and arrested the cell cycle; the presence of interleukin-6 did not overcome the Poly P-induced apoptosis. These observations suggest the relevance of Poly P to the humoral immune response and Poly P as a target for therapy of myeloma (119).

P_i TRANSPORT, POLY P, AND THE *PPK-PPX* OPERON

Phosphate (P_i), an essential nutrient, is often present in low amounts in the environment; organisms take up P_i by using one of several plasma or inner membrane transporters. *E. coli* possesses a number of P_i transporters, including the low-affinity, phosphate-inorganic transporters (PitA and PitB) and the high-affinity, phosphate-specific transporter (Pst). PitA transporter also operates the efflux of P_i and is energized by a PMF; a special energy requirement for Pst has also been noted (120, 121).

Several lines of evidence suggest involvement of Poly P and the *ppk-ppx* operon in P_i uptake and accumulation. A PitA-defective mutant has Poly P levels several times that of the WT. The rate of P_i uptake by the Pit system is reduced by half or more in a *ppk-ppx* deletion mutant, but uptake by the Pst system is unaffected. Attempts to construct a triple mutant lacking *pitA*, *pst*, and *ppk1* have failed, thus implying that PPK1 can serve if needed as an alternative to the Pit and Pst systems. In fact, overexpression of PPK1 doubled the rate of P_i uptake, indicative too that *ppx* expression in the operon provides a balance. In the *ppk* mutant of *Streptomyces lividans*, the need for P_i can be met by the overexpression of a Pst gene (122). Although the presence of a membrane complex of Ca-Poly P is known to enable the uptake of DNA, the mechanism whereby Poly P and PPK1 operate in P_i transport is still unknown.

MOTILITY

The operations responsible for the variety of bacterial movements are generally dependent at some stage on PPK and Poly P. The movements include swimming, swarming, and twitching as observed in *P. aeruginosa* (**Figure 8**). In the gliding by myxobacteria, the "social motility" resembles twitching in *P. aeruginosa* in that both depend on type IV pili. Motility is commonly a virulence factor and employed in stages of

development, such as the formation of biofilms, fruiting bodies, and spores.

In *V. cholerae*, the effect of the *ppk* mutation is marginal. However, the WT bacteria store massive amounts of Poly P, which enhance their capacity to survive in phosphate-limited, stressful aquatic environments. In the case of *H. pylori*, *ppk* mutants showed wide strain variations in phenotypic features, with motility among them.

Swimming and swarming both depend on polar flagella for their surface motility: on just one flagellum for swimming and on many for swarming. Twitching, as noted above, depends on pili. Barring defects in these structures in the *ppk* mutant, functional defects associated with a regulatory failure may be responsible.

The stationary-phase σ-factor, RpoS, is required for swimming and twitching motility as well as alginate production in *P. aeruginosa*. Because Poly P and/or PPK are required for the induction of *rpoS* gene expression in *E. coli*, and assuming the same to be true in *P. aeruginosa*, the swimming, swarming, and twitching motility defects in the *ppk* mutant may be explained in most cases on a functional rather than structural basis. In fact, the *ppk* mutants possess apparently normal flagella and pili under conditions favorable for swimming and twitching but fail to make an additional flagellum needed for swarming.

Defects in various forms of motility because of abnormal function of flagella and/or pili could arise from defects in chemotaxis (chemosensing through the chemoreceptors) and/or the cytoplasmic signal transduction system. CheY, the response regulator of the two-component chemotactic signal transduction system, is the central control site of signal transduction in bacterial chemotaxis. The relative level of phospho-CheY regulates the direction of flagellar rotation, either clockwise or counterclockwise, by interacting with the switch complex at the base of the flagellar motor (123).

For the reversal of flagellar rotation in swimming, chemotaxis and the chemotaxis signal transduction system are essential. In *E. coli*, the signal transduction system is essential for swarming motility, but traditional chemotaxis is not (124). As noted above, the twitching motility of *P. aeruginosa* and the social gliding motility of *M. xanthus* are essentially the same process; both are based on type IV pili (125). Chemotaxis and the chemotactic signal transduction system, operating through the Frz proteins, which are homologous to enteric Che proteins, are required for the WT pattern of social gliding motility in *M. xanthus* (126). In a search for genes affecting twitching motility in *P. aeruginosa*, two *cheY* homologs, *pilG* and *pilH*, were found that do not affect swimming. For reversing the flagellar rotation in swimming, a different *cheY* homolog is involved (127). *P. aeruginosa* possesses at least two distinct chemotaxis systems: One controls flagellar swimming and presumably swarming; the other supports type IV pili-based twitching.

How might Poly P and/or PPK affect motility through chemotaxis and the signal transduction system? Poly P might substitute for ATP in CheY phosphorylation or phospho-PPK might directly transfer phosphate to some CheY-like proteins (phosphorylation by cross talk), thus affecting the flagellum/pilus operations at a functional level. Poly P might also interfere with the cellular Ca^{2+} level to act directly on the flagellar motor. In fact, Poly P

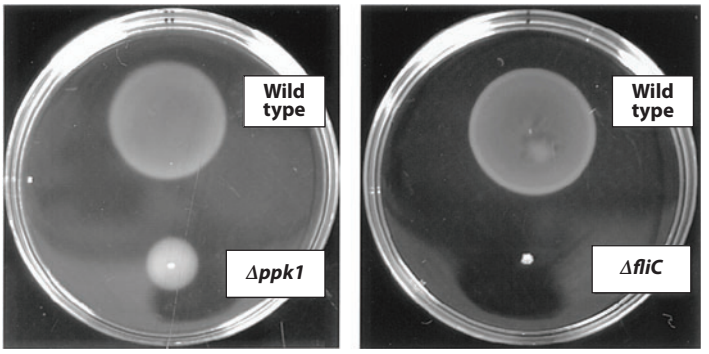

Figure 8

Swimming motility of *P. aeruginosa* PAO1, its *ppk1* mutant, and a *fliC* control strain derived from a *P. aeruginosa* PAK strain on a semisolid agarose plate. Cells were inoculated with a toothpick from an overnight Luria-Bertani broth agar plate onto a swim plate (tryptone broth plus 0.3% agarose) and photographed after a 14-h incubation at 30°C. Strains: wild type, PAO1; *Δppk1*, PAOM5; and *ΔfliC*, MS159 (62).

granules have been found at the base of the flagella in *H. pylori*.

Consistent with the abnormal function of flagella caused by improper chemotaxis and the reduced virulence in *S. typhimurium*, *Vibrio anguillarum*, and *Campylobacter jejuni*, the *ppk* mutant of *P. aeruginosa* is avirulent in a burned-mouse pathogenesis model, even though the mutant possesses normal flagella and pili.

PREDATOR-PREY RELATIONS

The role of Poly P and/or PPK in the contest between predator and prey can be observed in the outcome of interactions between many pairings of organisms. An instructive example is that of *P. aeruginosa* and the slime mold, *D. discoideum*. When WT *P. aeruginosa* was used in the plaque formation assay with the WT *Dictyostelium*, the bacteria were taken up by the mold, and the mold was killed, leaving the bacterial lawn intact. However, on a lawn of the *ppk1* mutant of *P. aeruginosa*, the WT *Dictyostelium* proved to be an effective predator as observed by the plaques formed (**Figure 9**). The *ppk1* mutant of *D. discoideum* also forms plaques on the bacterial mutant lawn, but they are much smaller than those formed by the WT mold (58). Thus, Poly P and/or PPK1 are crucial players in the contest between predator and prey. Attention is directed below to myxobacterium, one of the major predatory organisms.

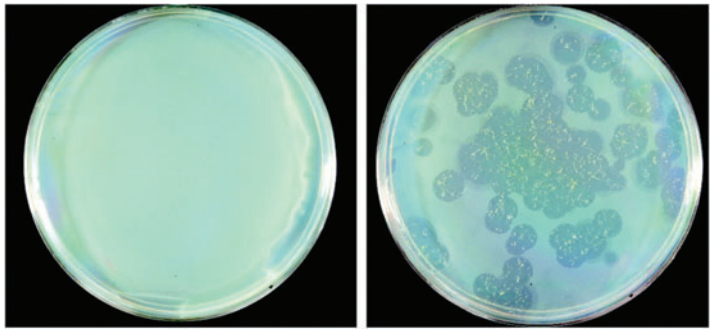

Figure 9

Plaque formation of *D. discoideum* cells on *P. aeruginosa* lawns. Wild-type (WT) cells were mixed with WT (*left*) and mutant (*right*) *P. aeruginosa*, plated on SM5 agar, and incubated at 22°C for 5 days (58).

MYXOCOCCUS XANTHUS: A "WOLF-PACK" BACTERIAL PREDATOR

M. xanthus is a gliding soil bacterium that exhibits social interactions and undergoes multicellular developmental cycles. During the vegetative phase, biofilms of *M. xanthus* consume other bacteria in a wolf-pack-like manner by producing several lytic enzymes and antibiotics. Upon starvation, cells at sufficiently high density initiate a multicellular developmental cycle whereby cells (10^5) aggregate into a mound-shaped fruiting body that contains thousands of resistant myxospores. The spherical myxospores germinate into rod-shaped vegetative cells when reexposed to a nutrient-rich medium.

Poly P is involved in the motility, the stages of development, and the predatory behavior. Null mutants of PPK1 are defective in gliding, a social motility that resembles twitching in *P. aeruginosa* and depends on polar type IV pili. Also defective in the mutant are the formation of fruiting bodies and the decreased number of spores in their germination (88). As an example, plaques of the mutant on a lawn of *Klebsiella aerogenes* are smaller in size (**Figure 10**).

Among other Poly P enzymes is the transferase (PAP) in *M. xanthus*, which converts adenylate to ADP and contributes to motility and biofilm formation but less so than PPK1. In response to starvation, a protein homologous to the bacterial Lon protease is expressed. This protein is essential for the expression of early developmental genes and may be dependent on Poly P for activation as proven in the case of Lon A in *E. coli* (128).

VIRULENCE FACTORS

Factors associated with bacterial virulence are generally expressed in the stationary phase of the growth cycle. At this stage, the need for PPK and Poly P for the survival of *E. coli* was first

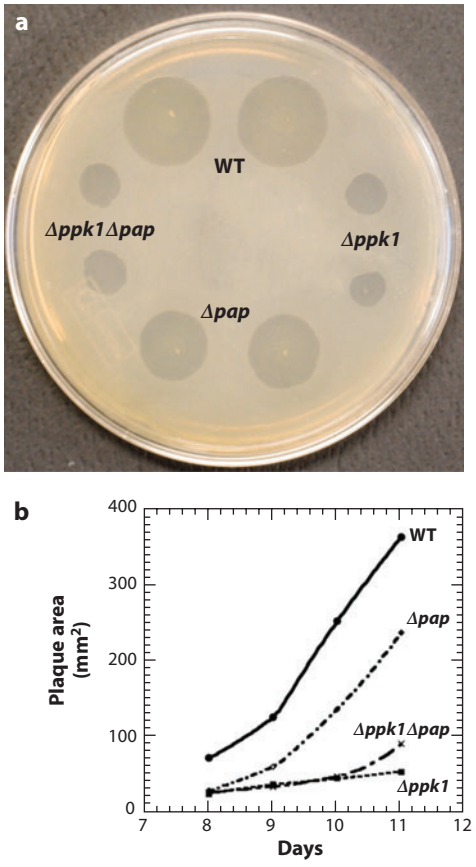

Figure 10

Predation on *K. aerogenes*. Overnight, *K. aerogenes* cultures were plated on nutrient agar. Wild type (WT) and mutants (*Δppk*, *Δpap*, and ***Δppk Δpap***) of *M. xanthus* were then spotted on these plates and incubated at 30°C. (*a*) Plaques on *K. aerogenes* lawn after 10 days. (*b*) Sizes of plaques during an 11-day period (88).

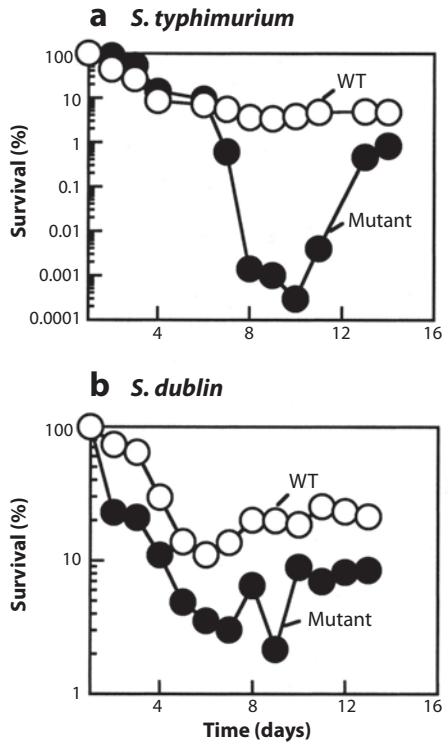

Figure 11

Long-term survival of wild type (WT) and mutants of *S. typhimurium* (*a*) and *S. dublin* (*b*) in Luria-Bertani broth medium (130).

observed (8, 129). *ppk1* mutants of *Salmonella enterica*, serovars *typhimurium* and *dublin*, showed a similar lack of long-term survival (**Figure 11**). Mutants in PPK1 of every pathogen tested also showed similar growth cycle effects and were defective in virulence factors that included motility, biofilm formation, responses to stringencies, tolerance to heat, acid, and desiccation, as well as resistance to inhibitors such as polymyxin (**Table 5**).

The pathogens tested included *Salmonella* sp., *Shigella* sp., *V. cholerae*, *H. pylori*, *P. aeruginosa*, *M. tuberculosis*, *and Neisseria meningitidis*. With *Salmonella* and *Shigella*, *ppk1* mutants were diminished in their invasiveness of epithelial cells (130). With *P. aeruginosa*, the deletion mutant turned out to be avirulent as tested by the burned-mouse model (63), and with *N. meningitidis* mutants, an increase in sensitivity to killing by human serum was observed (71).

Another measure of virulence is manifested in the predator-prey contest where the relative lack of PPK1 and Poly P is decisive in determining whether one or the other organism prevails.

ALGINATE, GTP, AND MUCOIDY IN PATHOGENESIS

P. aeruginosa in its mucoid state is a notorious pathogen, the cause of fatal pneumonia

Table 5 Defects in PPK1 mutants

Assay	Defect relative to wild type[a]			
	S. flexneri[b,c]	*S. typhimurium*	*S. dublin*	*E. coli*
Loss of PPK	+++	+++	+++	+++
Loss of PPX	+++	+++	+++	+++
Lack of Poly P	+++	+++	+++	+++
Growth in Luria-Bertani broth	+++	+	+	+
Loss of survival				
Short-term	+++	+	+	+
Long-term	NA	+++	+++	+++
Growth at low pH	+++	++	++	ND
Resistance to				
Acid	+++	+	++	ND
Heat	+++	++	+	+++
Polymyxin B resistance	ND	++	++	ND
Surface attachment	ND	+	++	++
Epithelial cell invasion	ND	++	ND	ND
Survival in macrophage	ND	++	ND	ND
Motility	NA	+++	+++	+++

[a]Defect has been graded from high (+++) to low (+) on the basis of data presented in results (130).
[b]*S. flexneri* loses viability in the short term, and the wild type is nonmotile.
[c]Abbreviations: NA, not applicable; ND, not determined; Poly P, polyphosphate; PPK, polyphosphate kinase; PPX, exopolyphosphatases.

in cystic fibrosis, and the scourge of burn victims. It is also among the best studied in Poly P metabolism. The mucoid is alginate, a secreted exopolysaccharide produced in a pathway in which GTP is a prominent precursor. The regulatory protein AlgR2 positively regulates not only nucleoside diphosphate kinase (Ndk), a source of GTP, but also ppGpp and Poly P (131). In the stationary phase, with depletion of nitrogen or phosphate, AlgR2 triggers the production of alginate along with stringent response molecules among which ppGpp and Poly P are important. Complementation of AlgR2 mutants by Ndk and other conditions that include Poly P reveal multiple adjustments that may restore alginate synthesis and the mucoid state. The PPK1 null mutant of *P. aeruginosa* failed to produce the exopolymer in biofilms (**Figure 12**).

Subsequent studies have identified PPK2 as the major Poly P–driven generator of GTP. Whereas PPK1 kinetically favors the synthesis of Poly P from ATP, PPK2 favors the reverse reaction, utilization of Poly P in the synthesis of GTP from GDP, by several hundredfold. PPK2 activity in *P. aeruginosa* becomes apparent at the end of exponential growth and early in the stationary phase as a prelude to the synthesis of alginate.

DEVELOPMENT: SPORULATION AND GERMINATION

Complex cellular events that encompass several developmental stages are invariably dependent on PPK and Poly P at some point. Among the examples is the sporulation of *B. cereus*. At least three principal enzymes and genes involved in the metabolism of Poly P are involved: PPK, PPX, and PAP. In the *ppk* mutant, Poly P levels are reduced to <5% of the WT; in the *ppx* mutant, PPK activity is elevated 10-fold and the accumulation of Poly P ~1000-fold (89). Each of the *Δppk*, *Δppx*, and *Δpap* mutants was defective in motility (**Figure 13**) and biofilm formation, but sporulation efficiency was impaired under standard conditions only in the *Δppx* mutant.

Notably the enzymes and genes in *B. cereus* are nearly identical to those in the very closely related pathogen *Bacillus anthracis* and, thus, may provide attractive targets for the treatment of anthrax.

DNA REPLICATION AND PHAGE PRODUCTION

Few reports have appeared on the role of PPK and Poly P in DNA replication. Yet it seems most probable that Poly P is involved in one or more of the multiple stages of a process so complex. Two studies in *E. coli* bear this out. One study involves a DNA polymerase in adaptive (error-prone) replication; the other is in the lytic growth of bacteriophages.

Adaptive mutations in *E. coli* occur in the error-prone replication carried out by DNA polymerase IV (Pol IV). Among the five or more kinds of DNA polymerase, Pol IV can synthesize DNA past a lesion in the template, like that produced by UV irradiation. In the course of such synthesis, Pol IV inserts an incorrect nucleotide at a higher frequency than does the principal Pol III polymerase. In doing so, mutations are suppressed throughout the genome at a rate 100 times greater than in a cell that is defective in either Pol IV or in PPK (132). Such error-prone (adaptive) replication may circumvent lethal errors associated with UV irradiation. Were a comparable mechanism identified in mammals, it might also prove important in carcinogenesis. Whether the defect in adaptation is due to a loss of replicative activity or an altered fidelity is unknown.

The observation that the lysogenic P1 phage failed as a vector in the transfer of genes (i.e., transduction) when the *E. coli* host was a mutant in *ppk* prompted studies of the basis of the defect. Although the adsorption of a WT P1 phage was unaffected in the PPK mutant, the spontaneous lysis (release or production) of phages was reduced by 100-fold. Defective particles accumulated in the mutant in large numbers and could be released upon UV treatment of the cell. Several deficiencies were

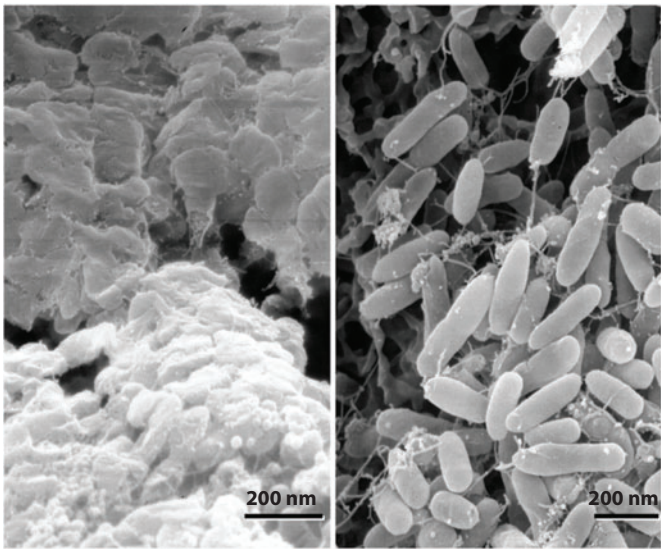

Figure 12

Scanning electron micrographs show mutant biofilms fail to produce exopolymer. *P. aeruginosa* wild type (*left*) and *ppk1* mutant (*right*) static biofilms were grown on nitrocellulose membranes in Luria-Bertani broth medium for 3 days (48).

demonstrable: One was a reduced expression of the *ssp* gene, encoded by the host and needed in transcriptional activation of P1 (133). Other defects observed were in the tail sheath of the mutant P1 progeny and in genomic alterations

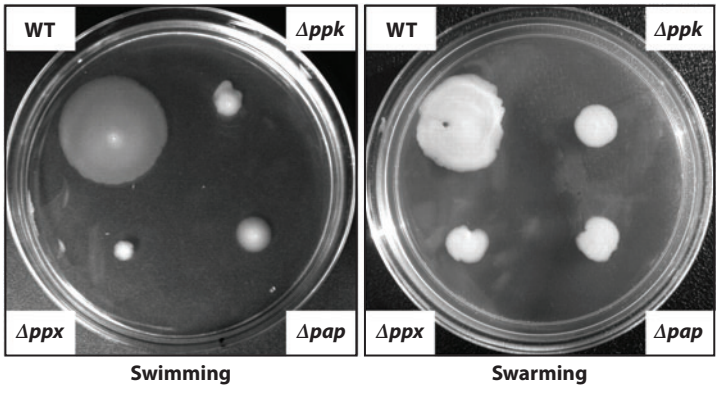

Figure 13

Swimming and swarming motility of wild-type *B. cereus* and the *Δppk*, *Δppx*, and *Δpap* mutants (89).

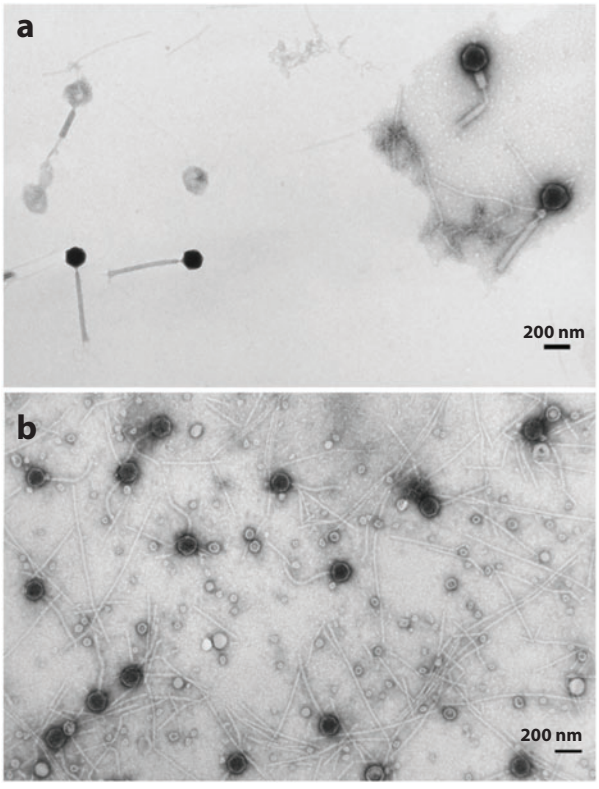

Figure 14

Transmission electron microscopy images of P1 (*a*) and mutant P1 (*b*) (133).

made evident in abnormal plaques produced on a lawn of WT host cells (**Figure 14**).

The filamentous phage fd (a member of the Ff single-stranded DNA phages that include M13 and f1) failed to produce plaques on a lawn of the *ppk* mutant. Although fd adsorbed to the F pilus, the DNA failed to enter the host cell. Inasmuch as the expression of TolA and the colicin A–mediated cell lysis were unaffected in the mutant, it is unlikely that the coreceptor function of TolA is at fault. The similarity of the F pilus and the type IV pili (involved in motility in which *ppk* mutants are defective) suggests that the defect may reside in the transfer of the phage from the pilus tip to the structures involved in fusion with the bacterial membrane and related PPK/Poly P components.

In view of the importance of Poly P in cellular metabolism, its role in phage replication may be widespread depending on the specific phage and host. As phages coevolve to use host components necessary for survival of the host, their replication will likely utilize Poly P in a variety of ways. The role of Poly P in the virulence and latency of viruses in eukaryotes also seems likely; an effect of Poly P on HIV adsorption to mammalian cells is of interest (134).

CHROMOSOME CONDENSATION

Of the many roles of Poly P in bacterial infrastructure, the organization of the nucleoid is especially notable. In *P. aeruginosa* mutants in *ppk1*, the nucleoid is compacted from a value of 41% of the cell area down to 26% (**Figure 15**). This may contribute to functional defects in motility, quorum sensing, biofilm formation, and virulence (48). These defects, in turn, may account for sensitivity to desiccation and the action of the β-lactam antibiotic, carbenicillin. Likely involved are the histone-like HU proteins, which are known to regulate gene

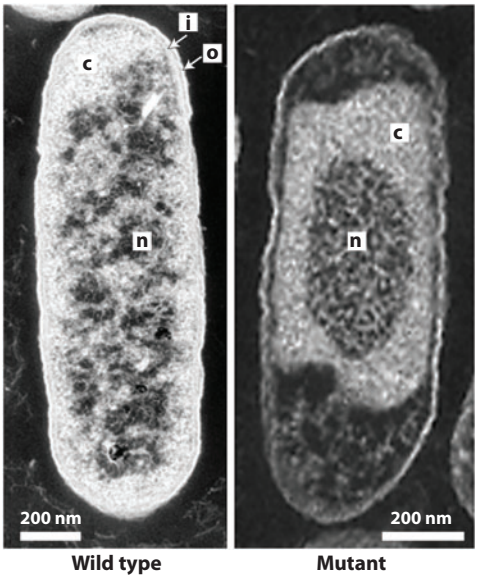

Figure 15

Transmission electron micrographs show compaction of the nucleoid and disruption of the cell envelope in *ppk1* mutant of *P. aeruginosa*. Wild type (*left*) and mutant (*right*) (48). Structure designations: n, nucleoid; c, cytoplasm; i, inner membrane; o, outer membrane.

expression (135, 136) and to bend and alter DNA at specific locations (137–140).

HU is one of the highly conserved and most abundant proteins (60,000 copies per cell) associated with the *E. coli* nucleoid. It stabilizes higher-order nucleoprotein complexes and belongs to a family of DNA architectural proteins. In vitro it shares the ability observed with histones to introduce negative supercoiling into relaxed DNA molecules in the presence of topoisomerase I (140). In vivo HU contributes to the maintenance of DNA superhelical density and to modulatation of topoisomerase I activity (141). In vitro genetically unlinked HUα and Huβ of *E. coli*, HUβ, HU-N, and HU-P proteins of *P. aeruginosa* bind strongly to Poly P. Binding of Poly P to each HU protein was analyzed by gel retardation assay. Each of these proteins could bind to Poly P more strongly than to linear double-stranded DNA. Poly P could also displace the HU proteins bound to DNA in vitro. A preference for Poly P of a specific chain length and the strength of binding differed among these HU proteins (K. Ishige & A. Kornberg, unpublished data). Thus, the specificity of interaction of Poly P with these basic proteins may determine the structure and function of the bacterial chromosome.

CYTOKINESIS AND CELL DIVISION

Cytokinesis includes the many stages from the mitotic division of the nucleus to the final splitting of the cell and from allocation of organelles and cytoplasm to the two daughter cells. These basic events apply broadly to both prokaryotic and eukaryotic cells.

DdPPK1 Mutant Cells Fail to Complete Cytokinesis

In addition to its functions in development and phagocytosis (58), DdPPK1 plays a role in the late stages of cytokinesis and cell division. Unlike WT cells, which were almost all mononucleated, about 30% of DdPPK1 and DdPPK2 mutant cells were multinucleated (60). WT cells completed cell division in less than 3.5 min after anaphase, whereas DdPPK1 mutant cells took longer; 16 of 39 cells observed failed to divide. Although the typical myosin II location in the cleavage furrow was seen in all cells, the furrow ingression in the mutant failed to progress to completion. After 10 to 25 (average, 17) min, daughter cells of the mutant fused and became multinucleated. Also observed were four binucleated parent cells that completed the first round of cytokinesis but failed to separate in the next round and became either two binucleated daughter cells or a mononucleated and a trinucleated cell. This phenotype of incomplete cleavage furrow ingression resembled that observed in a clathrin mutant of *D. discoideum* (142) and in a mutant of kinesin-like protein CHO1 of mammalian cells (143, 144). As with several other cellular events that depend on Poly P, the molecular basis for its actions in cytokinesis remains to be determined.

Localization of Kinesin 12 During the Cell Cycle in *Dictyostelium*

Several kinesin-like proteins have been identified in chromosomal segregation and cytokinesis (145, 146); among them the mitotic kinesin-like protein MKLP15 has been identified in humans as essential for cytokinesis. Its *Dictyostelium* homolog, named Kif12, traverses the mitotic spindle toward the midbody of the dividing daughter cells (147). Kif12 is localized inside the nucleus of interphase cells, exits the nucleus during mitosis, and is translocated to the mitotic spindle. Following mitosis, Kif12 is restored to the nuclear localization.

Other chromosomal passenger proteins likely play roles in chromosomal alignment, segregation, histone modification, and cytokinesis. Kif12 along with INCENP (148), AURORA B (149), and Survivin (150) are present in the nucleus during interphase (151). Kif12 is present in the nucleus of *D. discoideum* in a particulated network along with Poly P. However, in mutants lacking DdPPK1, Kif12 is localized on mitochondria in interphase cells

(M.R. Gómez-García & A. Kornberg, unpublished observations).

Unpublished data (from N.N. Rao, M.R. Gómez-García, & A. Kornberg) suggest that Kif12 is associated with Poly P in the nucleus during resting phase and associated with Poly P and mitochondria in stages of cytokinesis and cell division.

TRANSCRIPTION, MICROARRAYS, AND THE DEGRADOSOME IN POSTTRANSCRIPTIONAL REGULATION

Transcription and Microarrays

Mutants of *P. aeruginosa* or those of other gram-negative bacteria (including *E. coli*) that lack the major Poly P-synthesizing enzyme PPK1, exhibit a variety of defective pleiotrophic phenotypes (8, 63, 130). Because these defects are growth related, transcriptional analyses of mutants lacking PPK1 are useful. High-density, oligonucleotide microarrays are an important and versatile tool for genome-scale experimentation and are used to examine changes in global gene expression (152). Microarray analyses of stationary-phase cultures of WT and *ppk1* mutants of *P. aeruginosa* PAO1 grown in rich medium (Luria-Bertani broth) showed that, compared to WT, expressions of more than 450 genes of the *ppk1* mutant were decreased and that about 250 were increased by more than twofold (K. Ishige & A. Kornberg, unpublished observations). Similar results were found in initial experiments with the *E. coli* WT and *ppk1* mutant (C. Fraley & A. Kornberg, unpublished results).

In *P. aeruginosa*, among the 25 most severely repressed genes, 24 were related to quorum sensing (e.g., *paIL*, *mexGHI*, *rhlBA*, and *chiC*). A set of genes involved in the iron-starvation response system (e.g., *pvdS*, *prpL*, *aprXDEFA*, *pchGFEDCBA*, and *tonB*) was found among them (153). Another series of genes downregulated in the *ppk1* mutant is involved in the type III secretion system, responsible for the injection of exoenzymes into host cell cytosol, that causes rapid cell death (154, 155). Thus, numerous virulence genes either dependent or independent of quorum sensing are downregulated in the mutant, thus identifying Poly P as a "global regulator" of virulence. Notable among those genes upregulated are the σ^{54}-activated genes of amino acid and polyamine metabolism, nitrogen metabolism and transport, and the biogenesis genes of type 4 fimbriae involved in twitching motility.

Thus, PPK1 activity is involved in the transcriptional regulation, either directly or indirectly, of a large portion of the *P. aeruginosa* genome. In marked contrast, fewer than 20 genes are either increased or decreased in expression in the *P. aeruginosa ppk2* mutant, and the major role of PPK2 appears to be limited to the production of GTP from Poly P.

The Degradosome in Posttranscriptional Regulation

The rate of mRNA degradation is an important determinant of gene expression in prokaryotic and eukaryotic cells. In bacteria, mRNA decay is mediated by a degradosome, a multicomponent ribonucleolytic complex consisting of the RNA endonuclease RNase E, the 3′-5′ exoribonuclease, polynucleotide phosphorylase (PNPase), RhlB RNA helicase, and enolase. Two heat shock proteins, GroEL and DnaK, and PPK1 also are associated with degradosomes in substoichiometric amounts (156).

An *E. coli* strain deleted for the *ppk1* gene showed increased stability of the *ompA* mRNA. Poly P inhibited RNA degradation by the degradosome in vitro, an inhibition overcome by the addition of ADP, required for the conversion of Poly P to ATP by PPK1 in the degradosome (157). The proposed role of PPK1 in the degradosome is to maintain an appropriate microenvironment, removing inhibitory Poly P and NDPs and generating ATP. This hypothesis is substantiated by several earlier observations: (*a*) Poly P inhibits nucleic acid–modifying

enzymes, e.g., DNA ligase, restriction endonucleases, and DNA polymerase in vitro; (*b*) ADP is a potent inhibitor of PNPase, a component of the degradosome; and (*c*) regeneration of ATP by PPK1 is essential for RhlB helicase activity. In *Mycobacterium bovis* BCG and probably in *M. tuberculosis* H37Rv, the degradosome complex contains, in addition to RNase E and GroEL, two other copurified proteins: Poly P/ATP-NAD kinase and acetyltransferase (158). It was recently reported that, during the first adaptive phase of infection, the bacilli become sensitive to antibiotics and exhibit an increase in RNase E level. Poly P/ATP-NAD kinase catalyzes the reaction leading to the formation of NADP by ATP or Poly P. This enzyme in association with RNase E may play a regulatory role by helping adaptation to environmental changes. Given the central role of Poly P in survival during stringent growth conditions, it is conceivable that this Poly P–utilizing enzyme competes with RNase E in the degradosome to regulate RNA stability in mycobacteria.

STARVATION AND PROTEASE ACTIVATION

E. coli growing in a rich medium replete with the essential amino acids diverts its resources from making them into more useful metabolic pathways. Should the culture be shifted to a minimal medium, growth resumes only after a lag of hours, an interval needed for these amino acids to be made. They are generated by proteolytic action on the idled ribosomes, used first to make the very enzymes needed to synthesize these amino acids and later to make all the other proteins the cell needs for growth and survival. To cope with such stringency, the activation of protease by Poly P is a crucial event (**Figure 16**).

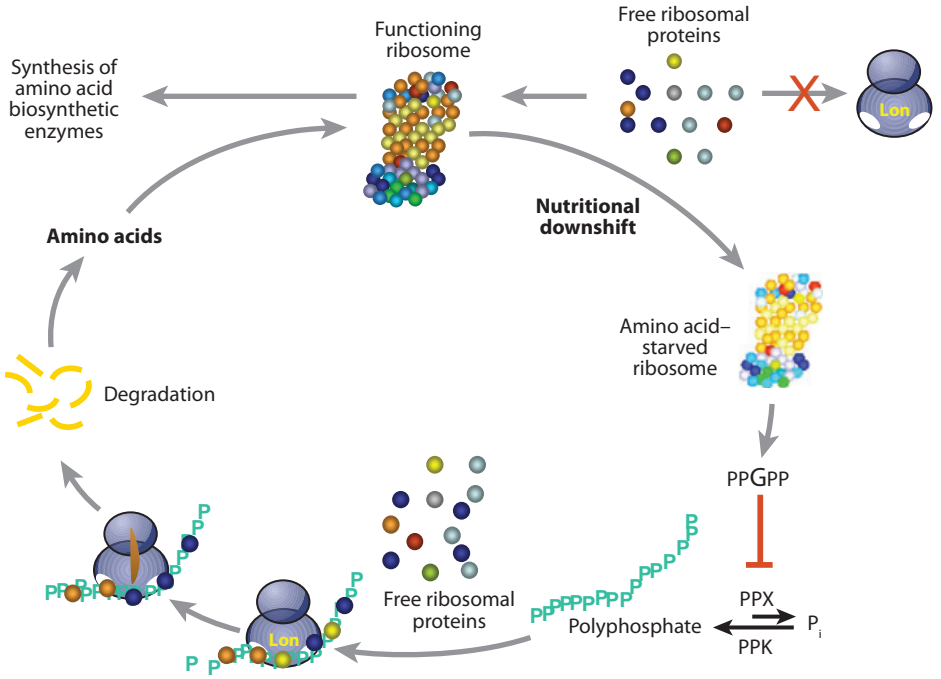

Figure 16

Polyphosphate kinase (Poly P)–dependent protein degradation is induced by nutrient depletion (183). Abbreviations: Lon, ATP-dependent protease; PPK, Poly P kinase; PPX1, exopolyphosphatase; ppGpp, guanosine tetraphosphate.

In response to a stringency such as the lack of one of the essential amino acids, the level of Poly P may increase more than 100-fold. Binding of Poly P to Lon A protease activates its degradation of proteins more than 20-fold, particularly certain proteins released from the inactive ribosomes. An amino acid pool is thereby generated for effective protein synthesis. Mutants in Lon A or in PPK fail to recover from the starvation imposed by a shift to a minimal medium. The Clp protease system, similar to Lon, may also be involved (128). The accumulation of Poly P depends on the stringent response system in which Rel A signals the generation of pppGpp, which among its many actions inhibits PPX, and thus promotes the accumulation of Poly P (8).

BONE FORMATION

Bone in vertebrate skeletons is composed of calcium phosphate in a state of crystalline apatite; noncrystalline calcium phosphate granules within the mineralizing bone and cartilage are Poly P (S. Omelon & M. Grynpas, personal communication). In the course of remodeling of bone by osteoblasts that make it and osteoclasts that degrade it, Poly P is a likely intermediate. Condensation of P_i into Poly P by kinases reduces P_i and Ca^{2+} levels and prevents excessive apatite formation. Although alkaline phosphatase has been regarded as the means of degrading Poly P to restore P_i levels, exopolyPases (PPX), abundant in osteoblasts, may be the preferred mechanism.

An in vitro study using osteoblast-like MC3T3-E1 cells revealed that Poly P could induce the expression of genes involved in bone differentiation and cell calcification (159). Poly P has also been shown to accelerate periodontal tissue regeneration, including alveolar bone formation, in rats that have artificial defects in periodontal tissue (160).

In implanting prosthetic devices in bone, as in the case of dental implants, various materials have been added at the site to facilitate the implant. Based on the known actions of Poly P as a dissolving and complexing agent and its likely participation in bone formation and remodeling, trials leading to the clinical use of Poly P seem warranted.

PLATELETS AND BLOOD COAGULATION

Poly P of 70–75 P_i residues has been identified in the dense granules of human platelets by staining with 4′,-6,diamidino-2-phenylindole, by NMR, and by gel electrophoresis. The granules are rich in Ca^{2+} and resemble acidocalcisomes that are widely distributed from bacteria to animal cells and judged to be responsible for the flux of Ca^{2+} into the cytosol (33).

Poly P release from activated platelets accelerates blood clotting by activating the contact pathway, including factor V, which, in turn, antagonizes the natural anticoagulant protein, the tissue factor pathway inhibitor. Poly P also delays clot lysis by enhancing a natural antifibrinolytic agent, the thrombin-activatable fibrinolysis inhibitor (161). Thus, Poly P released from platelets promotes clot formation and stability. Hydrolysis of Poly P by phosphatases promotes the inhibition of clotting and activation of fibrinolysis during wound healing.

MEDICAL APPLICATIONS

In an earlier review, we speculated on the basis of a high degree of sequence conservation among diverse bacterial species, that *ppk* knockouts of bacterial pathogens would render them avirulent (8). Because Poly P is essential for stringent and stationary-phase responses in *E. coli*, a similar role for Poly P is plausible in the expression of virulence factors that also appear in the stationary phase of pathogens and in the production of antibiotics by *Streptomyces* spp. (162).

During the past decade, the list of bacterial species with PPK1 has widened to nearly 100 and that of the major pathogens now includes some 20. The structure of PPK1 has been determined at atomic resolution including its unique active site for binding ATP. The way was made clear to prepare inhibitory

compounds that bind the site in addition to a large number already identified in screening available libraries of compounds. The absence of the PPK1 site in animal cells makes the toxicity of such inhibitory drugs unlikely.

PPK1: Target for the Treatment of Microbial Diseases

Of clinical significance has been the need for Poly P and PPK1 in the context of virulence of the pathogen in the host they invade. Mutants of *Salmonella* sp. showed drastically reduced acid tolerance, polymyxin B resistance, and diminished invasiveness in epithelial cells; survival in the macrophage was also severely compromised. Mutants of *Shigella flexneri*, the etiological agent of bacillary dysentery, were highly acid sensitive and failed in the Sereny test in guinea pigs. Mutants of *V. cholerae* were also acid sensitive and stationary-phase defective. Mutants of *N. meningitidis* in PPK1 were highly sensitive to killing by human sera; upon transformation back to PPK1$^+$, the serum resistance was restored.

With particular reference to *M. tuberculosis*, in addition to having the virtually identical PPK1 active site is the remarkable discovery that this bacterium needs PPK1 and Poly P for its very growth and survival; mutants in PPK1 fail in their responses to a variety of stringencies and stresses. When the synthesis of *ppk*1 in *M. tuberculosis* was attenuated (by expression of the gene in antisense orientation under the control of the *hsp60* promoter), it resulted in a decreased survival in macrophages (53).

Colonization by *H. pylori*

H. pylori is highly adapted to the human stomach, with polymorphism among clinical isolates. In mouse models of single infections, coinfections, and superinfections, a genetic variant evolved with a deletion of a 102-bp repeated sequence within the *ppk1* gene, involved in the physiological adaptation of the microbe to nutritional and environmental stresses (163). The deletion led to a higher enzymatic activity of PPK1 and a capacity to colonize mice, implying that PPK1 is an important virulence factor in *H. pylori*. In order to elucidate the role of PPK1 as a virulence factor, PPK1-deficient mutants were tested for colonization of the gastric mucosa (164). Absence of PPK1 expression did not impair in vitro growth of the PPK1 mutants but did decrease their motility, accumulation of Poly P, and ability to colonize. Remarkably, the importance of PPK1 to *H. pylori* differed among genetically diverse strains and phenotypes (165).

PPK2: Target for Treatment of *P. aeruginosa* Infections

Along with PPK1, PPK2 is widely conserved over a large range of bacteria. As observed with *P. aeruginosa*, PPK2 favors the Poly P–driven synthesis of a nucleoside triphosphate, in this case GTP from GDP. This reaction is in contrast with PPK1, which favors the synthesis of Poly P from ATP with no activity with GTP. In *P. aeruginosa*, the abundance of GTP drives the synthesis of the coenzyme that supplies the sugar for the alginate exopolysaccharide, a major factor in the pathogenesis of pneumonia in cystic fibrosis and the biofilms that coat burn victims.

DdPPK2: Target for Treatment of Protozoal Diseases

Mutants of *D. discoideum* lacking DdPPK1, the bacterial PPK homolog, maintain diminished but still significant levels of Poly P. Purification of the enzyme responsible for the synthesis of Poly P (DdPPK2) revealed several uncommon and unanticipated features. The enzyme is a polymer of 100 or more globular units, each a tetramer of three distinctive Arps similar to muscle actins in size, properties, and globular-filamentous structural transitions. Synthesis of a Poly P chain from ATP is concurrent at each step with elongation of DdPPK2 by one globular subunit.

DdPPK2 is localized in vacuoles, called acidocalcisomes, rich in calcium and Poly P and

responsible for the flux of calcium ions in the cell. Acidocalcisomes are ubiquitous from bacteria to humans and prominent in pathogenic protozoa (e.g., *T. cruzi*, *L. major*, *T. gondii*) and in the green algae, *Chlamydomonas* (34). Thus, DdPPK2 may provide a target for the treatment of protozoal diseases.

Periodontitis: Oral Gum Disease in Humans

Periodontitis is an inflammatory response of the gingival margin to mixed anaerobic bacterial infections. Among the organisms implicated, *Porphyromonas gingivalis* has attracted much attention. These organisms, expressing a variety of virulence factors, reside primarily in oral biofilm structures, commonly termed dental plaque (166). Both *P. gingivalis* and *Treponema denticola* are frequently associated in these subgingival plaques. When a mixture of both cultures was tested for attachment to an abiotic surface and visualized by confocal laser scanning microscopy, *P. gingivalis* formed synergistic biofilms with *T. denticola*. This property was dependent, in part, on the genes *fimA* and *ppk1*as well as the *usp* genes of *P. gingivalis*. Microarray and Northern blot analyses suggest that expression of the *ppk1* gene was required for maximal expression of the *uspA* gene (167). WT *P. gingivalis* accumulates short-chain Poly P; a mutant lacking Poly P (CW120 *ppk1)* was attenuated in biofilm formation on abiotic surfaces and failed to survive in the stationary phase. Complementation of the mutant with the *ppk* gene restored its biofilm formation and stationary-phase survival. Thus, PPK or Poly P is required for incorporation of *P. gingivalis* into subgingival plaque in humans.

Ocular Infections

P. aeruginosa can produce a severe corneal infection in wearers of soft contact lenses in less than 4 days. The keratitis can result in irreversible corneal scarring and even the loss of an eye (168). In a mouse model, *P. aeruginosa* PAOM5, an isogenic mutant of PAO1 deficient in PPK1, was significantly less virulent than either the WT or the mutant complemented with *ppk1* despite the ability to adhere to the corneal epithelium and grow in a minimal medium simulating tear film. The PPK1-deficient mutant also produced significantly less pyocyanin and may be more susceptible to oxidative stress.

INDUSTRIAL APPLICATIONS

The importance of Poly P in a variety of industrial applications, previously reviewed, deserves to be restated and reaffirmed. The number and variety of practical applications of Poly P have expanded even more than the basic knowledge of its chemistry and physiology. These applications include the microbial remediation of phosphate and toxic metals in wastewater treatment, the use of plants in phytoremediation, and the use of Poly P in the biomining of copper. Poly P has been used as a food additive for certain properties with virtually no cost and no health concerns. In biotechnology, it is supplied as a cheap source of high-energy phosphate to substitute for costly ATP.

In a number of major infectious diseases, identifying the enzyme responsible for Poly P synthesis has provided an attractive target for the rational design of therapeutic drugs. The superior function of biodegradable Poly P as an insulating fiber in construction and nonflammable clothing needs renewed attention as an alternative to the banned use of asbestos.

Environmental Remediation: Enhanced Biological Phosphate Removal

Population growth and intensive farming have led to a collapse of the natural phosphate cycles. Some 140 million tons of rock phosphate need to be recycled annually. Failing that, algal blooms destroy the use of bays, lakes, and waterways.

One method of phosphate removal is by precipitation with metal salts (e.g., alum, lime, or iron); the method is expensive and toxic and may require further removal of the heavy metal.

The alternative method, enhanced biological phospate removal, is more efficient, less costly, and produces a sludge waste that is more manageable. The processes for microbial phosphate removal and Poly P production from wastewaters have recently been reviewed (169). Current studies aim to obtain the most efficient microorganisms and to characterize the enzymes in the uptake of phosphate and the conversion to Poly P and its accumulation. Beyond that, the industry can benefit from better methods to release Poly P from the sludge and harvest by-products, such as methane.

The ability to concentrate diverse microbes is a top priority for sample collection methods that are used for emergency response and environmental monitoring when drinking water may be contaminated with an array of unknown dangerous microorganisms. Use of ultrafilters pretreated with different combinations of chemical dispersants and surfactants can significantly reduce the number of bacteria and viruses present in environmental sources, such as tap water. Because Poly P has a highly negative charge, minimizing the adhesion of microbes to filter surfaces, it has been used as a chemical dispersant in ultrafiltration for a rapid recovery of the microflora from drinking water (170). Poly P has also been used to decrease bacterial adhesion to soil (171) and to keep minerals in suspension during industrial processing.

Remediation of Mercury, Copper, and Uranium

Bacteria have been genetically engineered to take up and sequester heavy metals, thereby removing these toxic elements from the environment. As one example, toxic levels of phenylmercury as well as Hg^{2+} were sequestered in *E. coli* when transfected with plasmids that led to overproduction of Poly P and *mer* genes, which allowed efficient mercury uptake and its remediation in wastewater (172, 173). In a similar approach, a tobacco plant, *Nicotinia tabacum* stably transfected with plasmids expressing PPK1 are effective in the phytoremediation of mercury pollution (174, 175).

The copper tolerance of the thermoacidophilic archaeon, *Sulfolobus metallicus*, enables the organism to thrive in 200 mM copper sulfate facilitated by the accumulation of Poly P and the regulated efflux of copper, mediated by action of PPX. Applications to the biomining of copper now seem attractive (176).

The ubiquitous *P. aeruginosa* can withstand lethal doses of irradiation in the Poly P–facilitated removal of a uranyl ion. Overexpression of PPK1 in response to a variety of stimuli facilitates the removal of uranium by precipitation of uranyl phosphate (177).

Food Additive and Antimicrobials

Poly P as a food additive is widely used, especially in the meat and dairy industry, to enhance flavor, water binding, color retention, and emulsification, while retarding oxidative rancidity. Poly P also inhibits microbial growth, and in general, gram-positive bacteria are found to be more sensitive than gram-negative bacteria. *B. cereus*, an important food industry-related, toxin-producing, gram-positive spore former, which is genetically close to *B. anthracis*, is as sensitive to Poly P as are *Clostridium* spp. (178, 179). The effect of Poly P on *B. cereus* is growth related and concentration dependent. Higher concentrations of Poly P are bacteriocidal and cause cell lysis, whereas sublethal concentrations affect septum formation, leading to the formation of multinucleate, filamentous cells (179). The ability of Poly P to chelate cations is generally regarded as the cause for these observed inhibitory effects (180). Poly P is also implied in triggering leakage of Mg^{2+} from cells, loss of osmoregulation, and membrane damage in *Staphylococcus aureus*. Isolates of the gram-negative bacteria *Stenotrophomonas maltophilia* and *Acinetobacter* ssp. are naturally resistant to many classes of antibiotics. Using the disk diffusion technique, it was shown that membrane permeabilizers like Poly P increased susceptibility to a range of antibiotics, including imipenem, ciprofloxacin, tetracycline, and rifampicin. These effects are probably also due to the metal chelating properties of Poly P (181).

Regeneration of ATP and Other NTPs in Biotechnology

The cost of ATP for use as a phosphorylating agent on an industrial scale is prohibitive, as are the high costs of reagents, such as acetyl phosphate, phosphoenolpyruvate, and creatine phosphate, that are used in enzymatic ATP-regenerating systems. In their place, Poly P has been used to regenerate ATP with PPK immobilized on a column (90). In this system, a commercial form of Poly P costing less than $.50 a pound can provide ATP equivalents that would fetch over $2000. The Ndk activity of PPK1 has also been utilized in regenerating uridine triphosphate in the production of oligosaccharides by replacing phosphoenolpyruvate with Poly P (182) and in the synthesis of GTP by PPK2 in forming alginate, a precursor of mucoidy in pathogenesis.

The organism *C. glutamicum*, which contains only PPK2, is remarkable for accumulating 37% of its cell volume as a depot for Poly P and is used commercially in the annual production of 1.2 million tons of D-glutamate and 0.55 million tons of L-lysine.

Insulating Fiber

Phosphate fibers form bones and teeth. A calcium Poly P fiber has been synthesized with all the properties of asbestos, which could be a healthly safe substitute. But this product has been abandoned by its inventor, Monsanto Company, which cited fear of litigation from suits for alleged injuries from mineral fibers. Unfortunately, the decision to abandon Poly P fibers as an insulator also applies to its use for nonflammable infant sleepwear, hospital mattress covers, fabric for aircraft interiors, and numerous other applications.

PERSPECTIVES

We have dwelt in this review on the large number and variety of functions of Poly P discovered during the past decade. These furnish examples of Poly P roles that underlie pathways of cellular growth, survival, pathogenesis, development, and predation, as well as responses to all kinds of stress and stringency. Thus, the 2004 Perspective in "Inorganic Polyphosphate in the Origin and Survival of Species" (4) has been strengthened with still more examples.

Our approach to finding the multiple functions of Poly P has been to discover and characterize the enzymes that make and use it. Invariably, the source of Poly P in these reactions is ATP except in a single instance in which GTP can substitute. At several junctions in this review, the absence of such an enzyme in an organism, and the lack of a sequence homologous to encode it, has raised the possibility that Poly P can be made directly by a proton motive force (PMF) bypassing ATP as an intermediate.

Recall that prebiotically, Poly P was abundant on earth long before biologic molecules. By virtue of its capacity as a potent phosphorylating agent, Poly P is a plausible source of energy and phosphate in the early evolution of our phosphate world. As a potent chelator of metal ions, the variety of reactions that exploit Mg, Mn, Ca, Fe, and Co facilitate a multitude of reactions including the maintenance of ATP levels in a cell. As a metabolic regulator, Poly P has a unique role in the maintenance of homeostasis in the face of environmental stresses and stringencies. In mammary cancer cells, Poly P activates and regulates a key intermediate, mTOR, in the chain of events leading to their proliferation.

The possibility of PMF as a major source of Poly P needs to be studied in a defined, vesicular system in which PMF can be generated and regulated. Studies of the intact vesicle may provide the means to observe that ATP can be bypassed and PMF used directly in the synthesis of Poly P.

Despite these many advances, current textbooks and journals make hardly a mention of Poly P. This lack of attention to Poly P is another instance in which the power of fashion dictates pursuits in science. The dangers a scientist faces in working on an unpopular subject are reflected in the requirements for getting a job and grants, and the recognition obtained in organized meetings of the science community.

Among our efforts to attract attention to Poly P, we have demonstrated the essential roles of Poly P in the virulence of major diseases, such as dysentery, tuberculosis, and anthrax, as well as in apoptosis, in the proliferative aspects of cancer, in osteoporosis, and in aging. The selection and conservation of Poly P in every cell in nature will ultimately survive the current neglect of its scientific study.

FUTURE ISSUES

1. Although considerable progress has been made in the elucidation of metabolic functions of Poly P, transcriptional and translational regulation of specific genes involved needs to be studied.
2. The origin, fate, and function of short-chain Poly P present in abundance in eukaryotic cells need to be addressed.
3. The enzyme(s) responsible for the biosynthesis of Poly P in eukaryotes is virtually unknown and requires a close look.
4. Bioremediation, which involves removal of phosphate and toxic metals using bioengineered bacteria, needs to be studied and applied.
5. A rational design of succeessful therapeutic strategies involving Poly P kinase 1 and 2 with respect to drug-resistant bacterial pathogens, viz. *M. tuberculosis* and *P. aeruginosa*, deserves serious consideration.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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