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Genetically Altered Levels of Inorganic Polyphosphate in *Escherichia coli**

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The *ppk* gene encoding polyphosphate kinase (PPK), the enzyme in *Escherichia coli* that makes long chains of polyphosphate (polyP) reversibly from ATP, was disrupted by insertion of a kanamycin resistance gene. Expression of the exopolyphosphatase gene (*ppx*) immediately downstream of *ppk* in the operon was likewise disrupted. Cells were also transformed with a high-copy-number plasmid bearing *ppk*. Genetically altered polyP levels were estimated in cell extracts by the PPK conversion of ADP to ATP. PolyP levels (μg/10^{11} cells) near 2.0 were reduced in the *ppk*⁻-*ppx*⁻ mutants to 0.16 and increased more than 100-fold (*e.g.* 220) in cells transformed with multiple copies of *ppk*. Mutant cells, lacking the long polyP chains, showed a growth lag following dilution of a stationary-phase culture. PolyP-deficient cells exhibit a striking phenotype in their failure to survive in stationary phase and loss of resistance to heat (55 °C) and to oxidants (42 mM H_2O_2). High polyP levels are also associated with reduced survival.

Inorganic polyphosphate (polyP),[1] a long linear polymer of orthophosphates linked by high energy phosphoanhydride bonds, is widely distributed in bacteria, fungi, protozoa, plants, and mammals (1–3). Yet little is known about its cellular functions. Potential roles include: (i) energy source (1, 2), (ii) phosphate reservoir (1), (iii) donor for sugar and adenylate kinases (4–6), (iv) chelator for divalent cations (7, 8), (v) buffer for alkaline stress (9), (vi) regulator of transcription,[2] and (vii) component in competence for DNA entry and transformation (10).

A membrane-associated enzyme in *Escherichia coli*, polyphosphate kinase (PPK), catalyzes the synthesis of polyP from the terminal phosphate of ATP in a freely reversible reaction ($n\text{ATP} \leftrightarrow n\text{ADP} + \text{polyP}_n$) (11–13). Cloning of the *ppk* gene (14) also identified an adjacent gene, *ppx*, which encodes an exopolyphosphatase (PPX), an enzyme which processively releases orthophosphate from the termini of polyP in the reaction ($\text{polyP}_n \rightarrow \text{polyP}_{n-1} + \text{P}_i$) (15). Expression of *ppx* is dependent upon the *ppk* promoter, indicative of a polyphosphate operon (15).

In attempts to elucidate the functions of polyP, we have constructed mutants that fail to express *ppk* and *ppx* as well as cells which overproduce PPK. The consequences of altering the level of polyP 1,000-fold have been examined.

MATERIALS AND METHODS

Reagents—Sources were: ATP, ADP, creatine kinase, DNase I, and RNase IIIa, Boehringer Mannheim; creatine phosphate, MOPS, and polyP glass (type 65), Sigma; [α-^{32}P]dATP and [γ-^{32}P]ATP, Amersham Corp.; restriction endonucleases, New England Biolabs; Immobilon-N membrane, Millipore; and polyethyleneimine-cellulose F thin-layer chromatography plates, Merck.

Bacterial Strains, Plasmids, and Phages—*E. coli* K12 derivatives were: DH5α (F⁻, *supE44*, Δ*(lacZYA-argF)U169[φ80lacZΔM15]*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, *deoR*); SC864 (*F*⁻, *polA12*(ts), *thi-1*, *thr-1*, *leuB6*, *lacY1*, *fhuA21*, *supE44*, *fad751*::Tn*10*, λ⁻); *JM101* (Δ*(lac-proAB)*, *supE*, *thi-1/F′[traD36, proAB*⁺, *lacI*^{q}, *lacZΔM15]*; CF1648 (F⁻, λ⁻); NK6056 (Δ(*gpt-lac*)*5*, *purC80*::Tn*10*, *relA1*, *spoT1*, *thi-1*, λ⁻); *N3007* (λ⁻, *gua-26*::Tn*10* (in *guaA* or *guaB*), IN(*rrnD-rrnE*)1). DH5α was the host strain for plasmid preparations. SC864 was used to construct *ppk*⁻ mutants by inserting, through homologous recombination, the *kan* gene into *ppk* on the *E. coli* chromosome, as described below (Fig. 2). The mutated *ppk* genes were transferred into JM101 by P1 transduction (16); CA10 contains the *kan* gene in the same orientation as *ppk*, whereas CA11 contains *kan* in the opposite orientation. NK6056 and N3007 were used to map the integration site of *kan* on the chromosome. Phage λ 10H6 (14, 17) was from the Kohara λ library (17). Plasmid pBC8 (Figs. 2 and 4) was used as a probe for Southern blotting. Plasmid pBC29 used for polyP overexpression is a multicopy plasmid bearing *ppk* (15).

Survival Assays—Long-term survival of *E. coli* was assayed by growing cells in MOPS-buffered minimal medium (18) containing glucose (0.1%) as the carbon source and K_2HPO_4 (2 mM) as the P_i source. Cultures (3 ml) incubated at 37 °C for prolonged periods in glass test tubes (18 by 200 mm) were aerated by rotation in a New Brunswick gyratory wheel. Viable cell counts were determined by plating onto MOPS-buffered minimal agar medium containing 0.4% glucose and 2 mM K_2HPO_4; kanamycin (50 μg/ml) was added to the medium on which the mutant CA10 was plated.

For the heat-shock survival assay, cells were grown overnight (~20 h) in LB. The stationary-phase cells were washed and diluted in 0.9% NaCl to a density of about 5 × 10^3 cells/ml. Samples (2 ml) were put in glass tubes prewarmed to 55 °C and at times indicated, aliquots (0.1 ml) were plated directly on LB plates to determine viable cell numbers.

To test sensitivity to H_2O_2, cells were grown overnight (~20 h) in LB, washed, and resuspended in 0.9% NaCl to an OD_{540} of 1.0. H_2O_2 was added to a final concentration of 42 mM. At the times indicated, 0.1-ml samples were withdrawn, diluted immediately in 0.9% NaCl and plated onto LB plates to determine viable cell numbers.

Extraction of PolyP from E. coli—LB medium (1,500 ml) was inoculated to an optical density ($A_{600\,nm}$) of 0.05 with a sample of an overnight culture and incubated at 37 °C. Cells were harvested by centrifugation (6,000 × *g*, 7 min, 2 °C) when the culture reached mid-log phase growth. The resulting cell pellet was resuspended and lysed in ice-cold 2% trichloroacetic acid (0.6 ml); the suspension was maintained at 0 °C for 30 min with occasional vigorous mixing. Acid-insoluble material was collected by centrifugation (3 min, 14,000 × *g*, 2 °C), and the pellet was washed with ice-cold 67% acetone (1 ml) and collected by centrifugation, as above. The pellet was resuspended in 50 mM HEPES-KOH, pH 7.5 (0.5 ml), and the pH was adjusted to neutrality by the addition of 0.2 M KOH. $MgCl_2$ (5 mM), DNase I, and RNase IIIa (300 μg/ml of each) were added, and the mixture was incubated at 37 °C for 30 min. Proteinase

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[1] The abbreviations used are: polyP, polyphosphate; PPK, polyphosphate kinase; PPX, exopolyphosphatase; MOPS, 4-morpholinepropanesulfonic acid; kb, kilobase pair(s).

[2] A. Ishihama, personal communication.

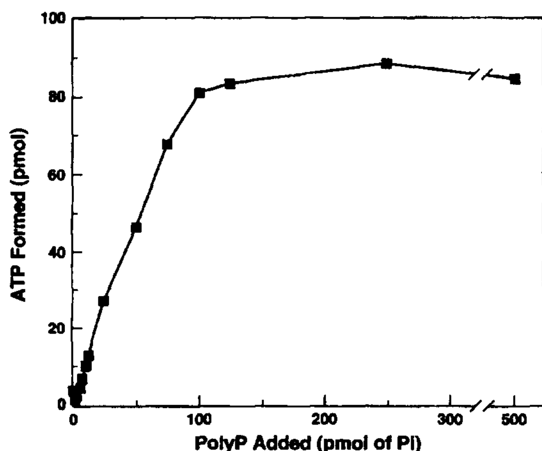

FIG. 1. **Enzymatic assay for polyP.** Synthetic polyP glass (type 65) solutions of known concentration (see "Materials and Methods") were incubated with [^{14}C-U]ADP and purified PPK. The level of ATP formed was determined as described (see "Materials and Methods").

K was added (600 μg/ml) and the incubation was continued for an additional 45 min. The mixture (approximately 1 ml) was extracted with phenol:chloroform (0.5 ml, 1:1, equilibrated with 0.1 M ammonium acetate), the phases were separated by centrifugation (5 min, 14,000 × *g*, room temperature), only the aqueous phase was removed and discarded. Additional portions of phenol:chloroform (0.2 ml) and 50 mM HEPES-KOH, pH 7.5 (0.5 ml), along with EDTA (to 10 mM) were added to the interfacial material and organic phase. The sample was mixed vigorously, incubated at 37 °C for 5 min, and the phases were separated by centrifugation, as above. The aqueous phase was removed and retained. Extraction of the organic phase was repeated three more times with 50 mM HEPES-KOH, pH 7.5, 10 mM EDTA (0.25 ml/extraction), and all of the aqueous phases containing EDTA were pooled. The pooled sample was treated twice with water-saturated chloroform (1 ml/extraction), followed by the addition of ice-cold *sec*-butanol (1.0 ml). The mixture was kept at −20 °C for 30 min or more, and the precipitated polyP was collected by centrifugation (15 min, 14,000 × *g*, 2 °C). The pellet was washed with ice-cold acetone (1 ml), dried under vacuum, and resuspended in water (0.2 ml).

Assay for PolyP—The ability to serve as a substrate for the PPK-catalyzed conversion of ADP to ATP was used as an assay to quantitate levels of polyP. The reaction mixture (10 μl) contained: 50 mM HEPES-KOH, pH 7.2, 40 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, and 11.6 μM [^{14}C-U]ADP (0.1 Ci/mmol), 2,000 units PPK, and polyP as indicated. The reaction (37 °C for 45 min) was terminated by chilling to 0 °C and the addition of ADP and ATP (to 5 mM of each). Aliquots were spotted onto a polyethyleneimine-cellulose F thin-layer chromatography plate; polyP, ADP, and ATP were resolved using 0.4 M LiCl, 1.0 M HCOOH as a solvent system and visualized with UV-irradiation. The radiolabel that remained at the origin and that which migrated with ADP, ATP, and the solvent front was quantitated by liquid scintillation counting. With ADP in excess, PPK catalyzed the almost complete consumption of polyP, yielding an equivalent amount of ATP (Fig. 1).

Other Procedures—PPK and PPX were assayed as described (14, 15). PPK (3 × 10^7 units/mg) was purified as described (14). [^{32}P]PolyP was synthesized with PPK from [γ-^{32}P]ATP and purified (15). The concentrations of polyP glass (type 65) solutions were determined by measuring total phosphate (19).

RESULTS

Disruption of the ppk Gene—A 9-kb fragment of *E. coli* DNA spanning both *ppk* and *ppx* was cloned from phage λ10H6 (14, 17) into the vector pBR322 (Fig. 2*A*). The *Eco*RI site near the middle of the fragment is approximately 0.3 kb downstream of the start codon for *ppk* (14). A 1.3-kb DNA fragment of plasmid pUC4K (Pharmacia LKB Biotechnology Inc.) containing the *kan* gene was inserted at this *Eco*RI site in either orientation relative to the *ppk* gene to produce plasmids pBC22 and pBC24 (Fig. 2*B*).

The wild-type *ppk* gene on the *E. coli* chromosome was replaced with the disrupted *ppk* gene by exploiting the requirement for functional DNA polymerase I to replicate plasmids with a *Col*E1 origin. A strain (SC864) with a temperature-sensitive DNA polymerase I was transformed with pBC22 and pBC24 at a permissive temperature (30 °C). In addition to containing the inserted gene for kanamycin resistance, the plasmids also confer resistance to ampicillin through their pBR322 component. During growth at the permissive temperature, plasmids may integrate into the host chromosome by recombination between homologous regions of the plasmid and the chromosome (integration), or a part of the chromosome may be replaced with the corresponding cloned DNA by a double crossover event (replacement) (Fig. 3). When such cells are shifted to the nonpermissive temperature (42 °C) for DNA polymerase I function, plasmids are lost. Only those cells with a *kan* gene inserted into their chromosome can survive exposure to kanamycin.

Ampicillin- and kanamycin-resistant transformants were selected at the permissive temperature of 30 °C. Cultures of transformants were grown overnight at 30 °C in LB medium (16) containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml). Aliquots of each culture were used to inoculate LB medium containing kanamycin (50 μg/ml) for overnight growth at 42 °C. Appropriate dilutions of the cultures were spread onto LB agar plates containing kanamycin (50 μg/ml), and the plates were incubated at 42 °C. The resulting kanamycin-resistant colonies were screened for sensitivity to ampicillin. Kanamycin-resistant, ampicillin-sensitive cells (*ppk::kan*) were isolated from SC864/pBC22 and SC864/pBC24, and the disrupted *ppk* genes were transferred to the strain JM101 by P1 transduction yielding strains CA10 and CA11, respectively.

Confirmation of Disrupted ppk Genes—A 4.4-kb *Sal*I fragment upstream of *ppk* was subcloned from λ10H6 into the vector pUC18, and the resulting plasmid, pBC8, was used as a probe for Southern blotting. The ppk^- mutants were examined by Southern blotting (Fig. 4). The radiolabeled probe pBC8 hybridized to a 4.4-kb *Sal*I fragment of genomic DNA from JM101 (Fig. 4*A*, *lane 1*), but not from CA10 or CA11. Instead, CA10 and CA11 yielded a 5.1-kb fragment (Fig. 4*A*, *lanes 2* and *3*) as expected from examination of their physical map (Fig. 3*B*). The smaller band in *lane 4* corresponds to vector plasmid pBR322. Since *Hin*dIII endonuclease digests the *kan* gene into 0.6- and 0.7-kb fragments, the orientation of the inserted *kan* gene relative to *ppk* was confirmed by Southern blotting of *Sal*I and *Hin*dIII-digested genomic DNA (Fig. 4*B*). Whereas the 4.4-kb fragment from JM101 was not affected by the additional digestion with *Hin*dIII (Fig. 4*B*, *lane 1*), the 5.1-kb fragments from CA10 and CA11 were altered, confirming the insertion of the *kan* gene into *ppk*; the size difference of the resulting fragments demonstrates that *kan* is in the same transcriptional orientation as *ppk* in CA10, whereas in the opposite orientation in CA11. Following a *Sal*I-*Hin*dIII digestion, the expected 0.9-kb fragment from CA10 and 1.0-kb fragment from CA11 were found to hybridize to pBC8 with a longer exposure of the gel (data not shown). Additionally, transduction frequencies with P1 phage revealed that the gene for kanamycin resistance in CA10 is closely linked to *guaAB* and *purC*, genetic markers close to *ppk* (20) on the *E. coli* chromosome (data not shown).

PPK and PPX Activities in Mutant CA10 and CA11 Cells—Lysates prepared from JM101, CA10, and CA11 cells were assayed for PPK and PPX activities (Table I). JM101 cells contained PPK and PPX in quantities comparable with other wild-type cells (14, 15). However, in CA10 and CA11, cells in which the *ppk* gene has been disrupted, the levels of both PPK and PPX were below the limit of detection. Immunoblot analysis using anti-PPK serum also failed to detect PPK in the ppk^- strains (Fig. 5); analysis with anti-PPX serum also confirmed

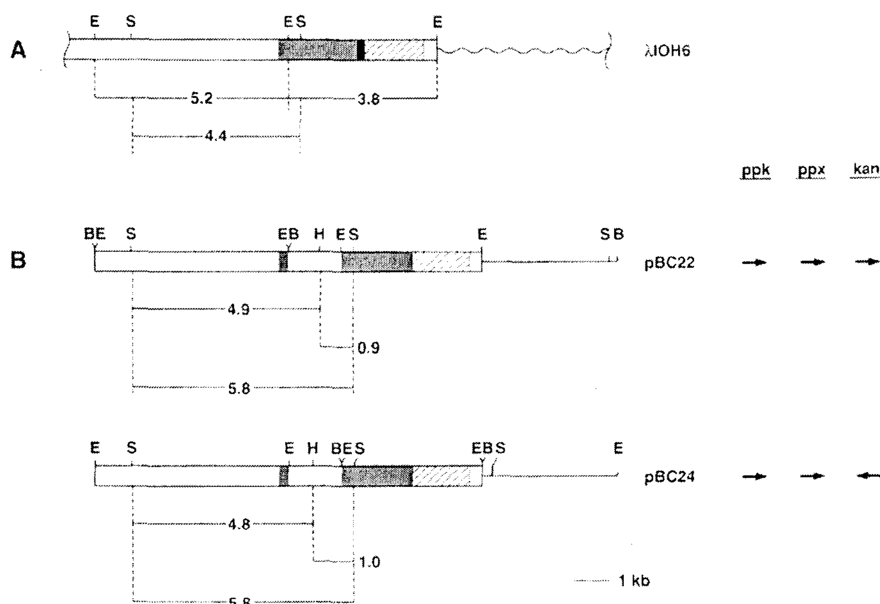

FIG. 2. **Maps of phage and plasmid DNAs.** *A*, a region of phage λ10H6 (14, 17). The *EcoRI* site shown at the junction of the *E. coli* DNA insert and the λ DNA come from the λ DNA. The *thick line* above the map shows the 4.4-kb *SalI* fragment cloned into pBC8. *B*, plasmids pBC22 and pBC24. Plasmids were constructed as described in the text. *Arrows* indicate the direction of transcription. DNA fragment lengths are shown in kilobase pairs. Symbols are: *unfilled box*, *E. coli* DNA; *filled box*, *ppk* gene; *cross-hatched box*, *ppx* gene; *stippled box*, 1.3-kb *kan* gene fragment; *wavy line*, λ DNA; *thin line*, vector pBR322 DNA. Abbreviations are: *B*, *BamHI*; *E*, *EcoRI*; *H*, *HindIII*; *S*, *SalI*.

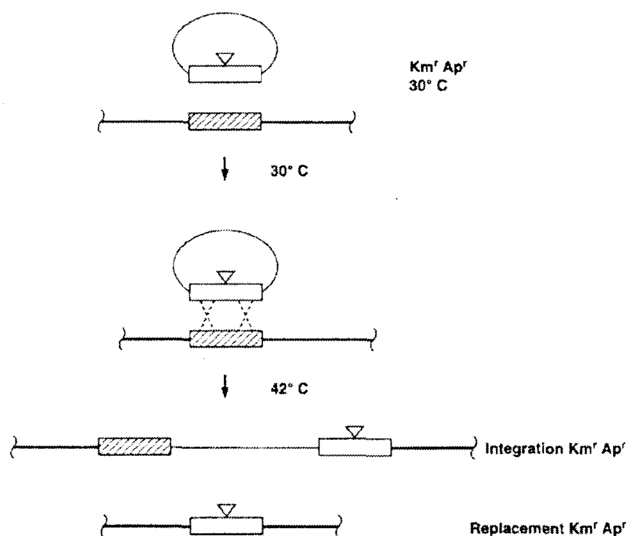

FIG. 3. **Strategy for the isolation of *ppk::kan* mutants.** Strains disrupted for *ppk* expression were isolated as described in the text. Symbols used are: *unfilled box*, cloned *E. coli* chromosomal DNA; *cross-hatched box*, region of the *E. coli* chromosome corresponding to the cloned DNA; ▽, *kan* gene inserted into the cloned *ppk* gene; *thick line*, bacterial chromosome; *thin line*, vector pBR322; Km_r, kanamycin resistance; Ap_r, ampicillin resistance; Ap_s, ampicillin sensitivity.

FIG. 4. **Southern blot analysis of *ppk*⁻ mutant genomic DNA.** *E. coli* genomic DNA was prepared from the *ppk*⁺ strain (JM101) and the *ppk*⁻ mutant strains (CA10 and CA11) as described (30). Genomic DNA and plasmid pBC8 were digested with: *SalI* (*A*) or *SalI* and *HindIII* endonucleases. Resulting fragments were separated by electrophoresis (*B*) through an agarose gel (1%), transferred to an Immobilon-N filter, and probed with radiolabeled pBC8 DNA. *Lane 1*, JM101 genomic DNA; *lane 2*, CA10 genomic DNA; *lane 3*, CA11 genomic DNA; *lane 4*, plasmid pBC8 DNA.

the absence of PPX (data not shown).

PolyP Content in Wild-type Cells, ppk⁻,ppx⁻ Mutants, and Cells Overproducing PPK—PolyP was extracted from cells with wild-type (JM101 and JM101/pUC18) and mutant (CA10 and CA11) levels of PPK and PPX and with overproduced (JM101/pBC29) (15) levels of PPK. The extraction procedure separated polyP from other phosphate-containing compounds in the cell lysate by its insolubility in cold acid and organic solvents, resistance to nucleases and proteases, and chelation by Mg^{2+} (see "Materials and Methods"). [^{32}P]PolyP, added to a portion of the initial cell lysates as a marker, was recovered with a yield of 90% or greater.

The polyP content of extracts prepared from the various cell types was measured using the PPK conversion of ADP to ATP, as described above. Cellular polyP concentration was about 2.0 μg/10^{11} cells in the wild type cells (JM101) and wild-type cells transformed with the vector plasmid (Table II). Another *E. coli* wild-type strain CF1648 contained 0.44 μg of cellular polyP. Cells overexpressing PPK had polyP at levels over 100-fold

TABLE I
Lysates from ppk⁻ strains lack PPK and PPX activities

Strains (JM101, CA10, and CA11) were grown in LB medium at 37 °C to an optical density ($A_{600\ nm}$) of 1. Cells were harvested by centrifugation and lysed as described (14, 15). PPK activity was measured for the sonicated lysate pellet fraction (14), and PPX activity was determined for the soluble lysate fraction (15).

Strain	Activities of: PPK	PPX
	units × 10⁻³/g cell	
JM101	320	290
CA10	<3.0	<5.3
CA11	<2.4	<3.8

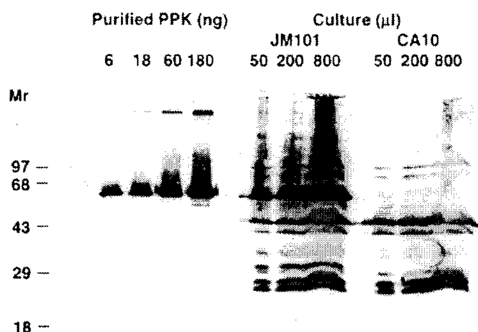

FIG. 5. **Cellular abundance of PPK.** Cells (JM101, CA10) were grown at 37 °C in LB medium to an optical density ($OD_{600\ nm}$) of 1.8. Total cell protein was precipitated from 1.3 ml of the culture with trichloroacetic acid (10% final concentration). The precipitate was harvested in a Microfuge, washed twice with 1 ml of ice-cold acetone, and solubilized in SDS-PAGE buffer. Samples were subjected to SDS-PAGE (15%). PPK was determined by immunoblotting with PPK antiserum. Purified PPK served as a standard.

TABLE II
PolyP content of cells with altered expression of ppk and ppx

Extracts enriched for polyP were prepared (see "Materials and Methods") and their polyP content was determined as for solutions of polyP glass (Fig. 1).

Strain	Genotype	PolyP content[a]
		µg/10¹¹ cell
JM101	Wild type	2.00
JM101/pUC18	Wild type	2.25
CA10	*ppk⁻,ppx⁻*	0.16
CA11	*ppk⁻,ppx⁻*	0.16
JM101/pBC29	*ppk* plasmid	220

[a] Assays of PolyP extracted from three different cultures were averaged.

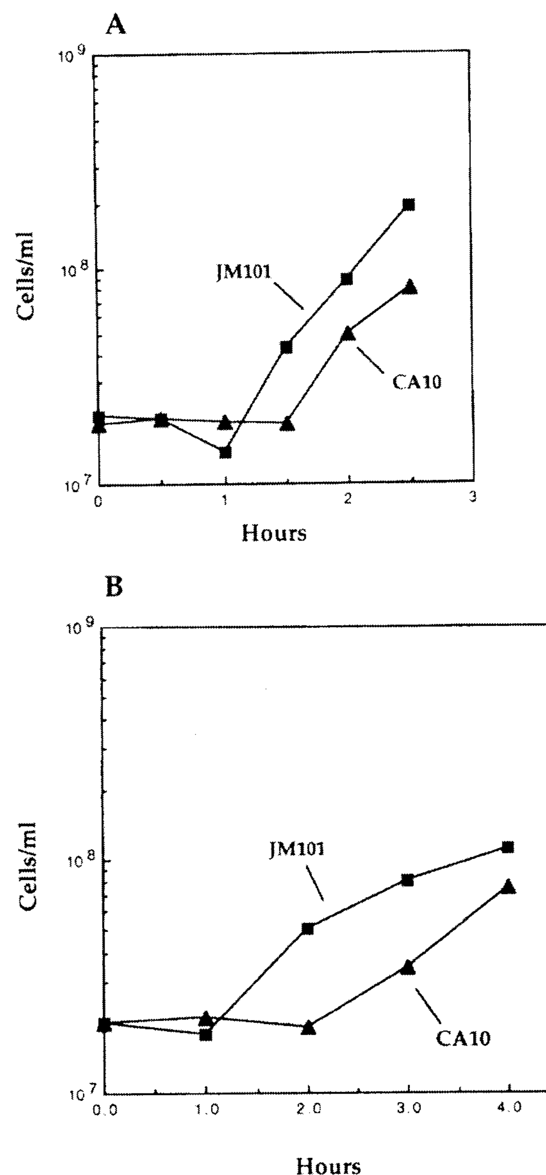

FIG. 6. **Growth of wild-type cells and mutants lacking long chain polyP.** Cells (JM101 and CA10) were grown in the indicated media to saturation. Prewarmed (37 °C) media were inoculated with samples of the stationary cultures and incubated at 37 °C. Appropriate dilutions of the cultures were plated onto media to monitor growth. Media were: *A*, LB (16) and *B*, M9 + 0.2% glucose (16).

higher than wild-type cells. PolyP levels in mutant cells (CA10 and CA11) that lacked a functional *ppk* and *ppx* operon were 0.16 µg/10¹¹ cells. The extracts from these mutant cells were not inhibitory to the PPK-catalyzed production of ATP, in that a mixture of CA10 extract and synthetically prepared polyP was indistinguishable from that of polyP alone (data not shown). Preliminary analysis of the polyP from the mutant cells indicated that they were predominantly short chain polymers with an average chain length of 60 (data not shown). It is likely that these short chain polyP are synthesized by an independent pathway.

Cells Deficient in PolyP Exhibit a Growth Lag—When JM101 (PPK^+) and CA10 (PPK^-) cells were grown on minimal medium (M9 + 0.2% glucose) plates (16), the mutant cells showed slow growth (small colony size) during the early stages of the incubation (data not shown). However, with further growth the difference in colony size decreased. The initial growth of the two cell types was measured following the inoculation of various media with stationary-phase cultures. In a rich medium (LB), the cell number for wild-type cells started to increase after 1 h, whereas the growth lag for mutant cells was reproducibly longer (Fig. 6*A*); this lag was also observed using a minimal glucose medium (Fig. 6*B*).

Effects of PolyP Deficiency on Long-term Survival in Stationary-phase, Thermal Resistance, and H_2O_2 Resistance—*E. coli* mutant strain CA10 which lacks PPK and PPX exhibited reduced survival in stationary phase (Fig. 7*A*). After 2 days in stationary phase in minimal medium with a limited carbon source (0.1% glucose), the viability of the mutants dropped to ~7% of the original value (Fig. 7*A*). The corresponding wild-type strain (JM101) did not show any significant loss in viability during this period. In addition to the loss in viability, a

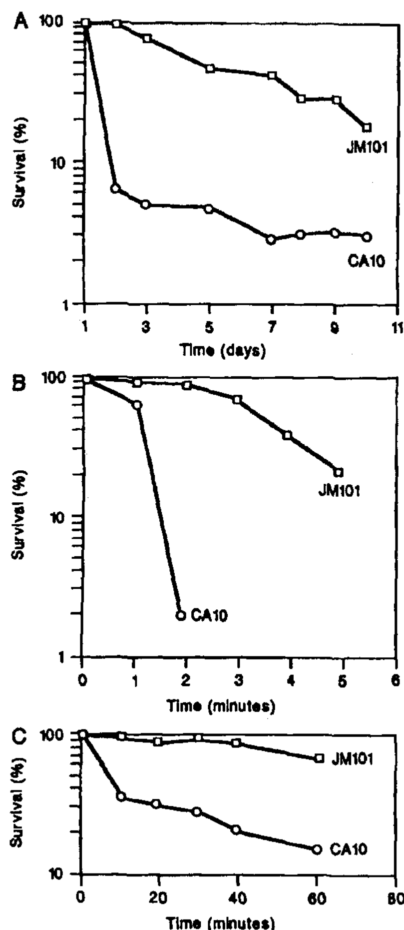

FIG. 7. **Physiological tests.** *A*, long-term survival of wild-type *E. coli* (JM101) and the polyP-deficient mutant (CA10) in MOPS-buffered minimal medium with limited glucose (0.1%, w/v). Percent survival represents the viable cell number at each time point divided by the viable cell number of the same culture after 24 h in stationary phase. Similar results were obtained in three experiments. *B*, heat-shock survival. Cells were grown overnight (~20 h) at 37 °C in LB medium, exposed to 55 °C, and the viability determined by plating on LB agar; 100% is equivalent to ~5 x 10^3 cells/ml. *C*, H_2O_2 sensitivity; stationary phase cultures were exposed to 42 mM H_2O_2, and viable cell numbers were determined as described under "Materials and Methods"; 100% corresponds to the viable cell number determined immediately before the addition of H_2O_2.

stable small colony phenotype emerged from stationary-phase CA10 cells after 48 h in stationary phase (data not shown). During survival in stationary phase or starvation, *E. coli* cells acquire rpoS (kat F)-mediated resistance to multiple stresses, including heat and oxidants (25–28). When polyP-deficient cells (CA10) were held at 55 °C for 2 min, only 2% survived, as compared with about 90% of the wild type (JM101) (Fig. 7*B*). Similarly, of the polyP-deficient *E. coli* cells in stationary phase exposed to 42 mM H_2O_2, only 40% survived as compared with about 85% of the wild type (Fig. 7*C*).

DISCUSSION

The ease with which orthophosphate condenses to form inorganic polyphosphate (polyP), a polymer linked by phosphoanhydride bonds, argues for a prominent role for the energy-rich compound in prebiotic evolution. In view of the ubiquity and abundance of polyP throughout nature, presumptions of multiple roles for this molecular fossil are reasonable, but exactly what these are must vary depending on where and when polyP serves in cellular functions.

In our approach to identify and examine polyP functions, we have purified enzymes responsible for the synthesis and utilization of polyP. The homogeneous enzyme provides a route to the discovery of the gene which encodes it and a means of modulating gene expression from depletion to overproduction of the enzyme. Phenotypes created by this "reverse genetics" may supply clues to the physiologic functions of polyP in cellular growth, metabolism, and development. Often unappreciated is the immediate utility of the purified enzyme as a reagent to prepare labeled well defined substrates and as an analytical tool to determine the features and abundance of polyP in extracts of various cells and organisms.

Previously, we identified the *E. coli* genes *ppk* for a polyP kinase (PPK) (14) and *ppx* for an PPX (15). These adjacent genes form an operon, the functions of which in polyP metabolism needs to be explored. To this end, we have constructed the mutant strains, reported in this study, in which the expression of *ppk* and *ppx* have been disrupted (Table I); overexpression of these genes, introduced in high copy number plasmids, has been obtained (14, 15). By these manipulations, the levels of polyP have been reduced more than 10-fold in the mutants and raised about 100-fold in the overproducer (Table II), an overall spread in the level of 1,000-fold.

The mutant cells are viable and show no striking phenotype as judged by growth in various media. Rates do not differ from wild-type cells in rich media at temperatures between 23 and 42 °C, in media limited in carbon (16) or in phosphate (21), at high ionic strength (*e.g.* 330–800 mM) or at decreased oxygen tensions (22). Nor were any growth rate differences observed between wild-type, mutant, and overproducer cells in a minimal medium containing a nonfermentable carbon source (*e.g.* succinate) or in the presence of dinitrophenol or azide at levels sufficient to reduce cellular ATP. The polyP content of wild-type cells grown on succinate was also similar to that of cells grown on glucose.

The most suggestive indications of a deficiency in the *ppk* mutant are in the adjustments made in response to nutrient deprivation and for survival in the stationary phase. Guanosine pentaphosphate hydrolase, one of the two enzymes (the other being RelA which synthesizes pppGpp) essential for making ppGpp in response to the stringency of amino acid starvation (23), has unexpectedly turned out to be a potent exopolyphosphatase, different from PPX (24). Furthermore, polyP levels increased 10-fold or more in cells treated with serine hydroximate, an amino acid analog that induces (p)ppGpp production.[3] How polyP is involved in regulating promoter selection in this and other circumstances remains to be determined.

The *ppk* mutant is impaired in responses to stress and deprivation. There is a striking lability to heat and to hydrogen peroxide; survival in the stationary phase is also affected (Fig. 7). After the second day in a minimal medium with a limited carbon source at 37 °C, there is not only a greater loss of viability in the mutant strain, but also the emergence of a small colony variant. These and other changes are suggestive of a selection for *rpo*S (*kat*F) alleles that direct patterns of gene expression essential for adjustments to remaining viable in the stationary phase (25–28). Cells with elevated polyP levels (JM101/pBC29, Table II) attained only one doubling in cell density when grown in minimal medium. The viable cell number at this stage was less than 4% of the wild type at stationary phase (data not shown).

PolyP has been identified as a component of a complex with polyhydroxybutyrate and Ca^{2+} in the membrane of bacteria competent for DNA transformation (10). The mutant strain with reduced levels of polyP can still acquire competence, al-

[3] E. Crooke and A. Kornberg, unpublished data.

though with less efficiency than the wild type.[4] The competent mutant strains do possess a chloroform-extractable polyP chain of about 60 residues, presumably synthesized by a pathway other than PPK.[5]

A remarkable ecological contribution made by PPK is in the bacterial removal of phosphate which pollutes waterways and causes algal blooms. In current sanitary engineering practice, an aerobic fermentation fixes phosphate in ATP, which is then converted by PPK to polyP and removed with the bacterial sludge (29). By transforming one of the bacterial strains with a high copy number plasmid bearing the *ppk* gene, phosphate removal from the surrounding medium became far more rapid and complete.[6] With a similar plasmid bearing both the *ppk* and *ppx* genes, overproduction of the PPX counteracts the effectiveness of PPK, a clear demonstration of the actions *in vivo* of each of these enzymes.

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[5] C. E. Castuma, R. Reusch, and A. Kornberg, unpublished data.

[6] H. Ohtake, personal communication.