Genetically Altered Levels of Inorganic Polyphosphate in
Escherichia coli*

Elliott Crooke†, Massahiro Akiyama, Narayana N. Rao, and Arthur Kornberg
From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

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 activity assay which measures ADP to ATP. PolyP levels (µg/10^6 cells) were near 2.0 were reduced in the ppk−ppx− mutants to 0.18 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 (85 °C) and to oxidants (42 mM H₂O₂). 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, 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 (nATP + nADP + polyP) (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 (polyPn−→polyPn−1 + P). 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; [γ-32P]ATP and [α-32P]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, xgalZ3C-argF) [U166 (80lacZAM15), red-I7, hsdR17, recA1, endA1, gyrA96, thi-1, relA1, deoR); SC864 (F−, polA125a, thi-1, thr-1, leuB6, lacY1, f1 aux2, supE44, f1d51, Tn10, λ−); JM101 (lac− pro− adk− recE− thi−1 purC− leuB− lacY1 f1 aux2, supE44, f1d51, Tn10, λ−); SC864 (X-gal-lac-Z, purC− lacY1 f1 aux2, supE44, f1d51, Tn10, λ−); SC864 (X-gal-lac-Z, purC− lacY1 f1 aux2, supE44, f1d51, Tn10, λ−)

DH5α was the host strain for plasmid preparations. SC864 was used to construct ppk− mutants by inserting, through homologous recombination, the kon 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 kon gene in the same orientation as ppk, whereas CA11 contains kon in the opposite orientation. LK6056 and N3007 were used to map the integration site of kon 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 multi-copy 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 K₂HPO₄ (2 mM) as the P 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 shaker. Viable cell counts were determined by plating onto MOPS-buffered minimal agar medium containing 0.4% glucose and 2 mM K₂HPO₄; 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 (about 20 h) in LB. The stationary-phase cells were washed and diluted in 0.9% NaCl to a density of about 5 x 10⁶ 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 H₂O₂, cells were grown overnight (about 20 h) in LB, washed, and resuspended in 0.3% NaCl to an OD₆₀₀ of 1.0. H₂O₂ 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 (A₆₀₀) of 0.05 with a sample of an overnight culture and incubated at 37 °C. Cells were harvested by centrifugation (6,000 x 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 x 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 mM KOH. MgCl₂ (65 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

*This work was supported in part by grants from the National Institutes of Health and the National Science Foundation (to A. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Supported by American Cancer Society, California Division Senior Postdoctoral Fellowship 8-26-91. Present address: Dept. of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, D. C. 20007.

‡Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

The abbreviations used are: polyP, polyphosphate; PPK, polyphosphate kinase; PPX, exopolyphosphatase; MOPS, 4-morpholinepropane-sulfonic acid; kb, kilobase pairs.

‡A. Ishihama, personal communication.
RESULTS

Disruption of the ppk Gene—A 9-kb fragment of E. coli DNA spanning both ppk and pxk was cloned from phage λ10H6 (14, 17) into the vector pBR322 (Fig. 2A). The EcoRI 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 \textit{kan} gene was inserted at this EcoRI site in either orientation relative to the \textit{ppk} gene to produce plasmids pBC22 and pBC24 (Fig. 2B).

The wild-type \textit{ppk} gene on the \textit{E. coli} chromosome was replaced with the disrupted \textit{ppk} gene by exploiting the requirement for functional DNA polymerase I to replicate plasmids with a ColE1 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 \textit{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 \mu g/ml) and kanamycin (50 \mu g/ml). Aliquots of each culture were used to inoculate LB medium containing kanamycin (50 \mu g/ml) for overnight growth at 42°C. Appropriate dilutions of the cultures were spread onto LB agar plates containing kanamycin (50 \mu g/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 (\textit{ppk}:\textit{kan}) were isolated from SC864/pBC22 and SC864/pBC24, and the disrupted \textit{ppk} genes were transferred to the strain JM101 by P1 transduction yielding strains CA10 and CA11, respectively.

Confirmation of Disrupted \textit{ppk} Genes—A 4.4-kb \textit{SalI} fragment upstream of \textit{ppk} was subcloned from λ10H6 into the vector pUC18, and the resulting plasmid, pBC8, was used as a probe for Southern blotting. The \textit{ppk}− mutants were examined by Southern blotting (Fig. 4). The radiolabeled probe pBC8 hybridized to a 4.4-kb \textit{SalI} fragment of genomic DNA from JM101 (Fig. 4A, lane 1), but not from CA10 or CA11. Instead, CA10 and CA11 yielded a 5.1-kb fragment (Fig. 4A, lanes 2 and 3) as expected from examination of their physical map (Fig. 3B). The smaller band in lane 4 corresponds to vector plasmid pBR322. Since \textit{HindIII} endonuclease digests the \textit{kan} gene into 0.6- and 0.7-kb fragments, the orientation of the inserted \textit{kan} gene relative to \textit{ppk} was confirmed by Southern blotting of \textit{SalI} and \textit{HindIII}-digested genomic DNA (Fig. 4B). Whereas the 4.4-kb fragment from JM101 was not affected by the additional digestion with \textit{HindIII} (Fig. 4B, lane 1), the 5.1-kb fragments from CA10 and CA11 were altered, confirming the insertion of the \textit{kan} gene into \textit{ppk}; the size difference of the resulting fragments demonstrates that \textit{kan} is in the same transcriptional orientation as \textit{ppk} in CA10, whereas in the opposite orientation in CA11. Following a \textit{SalI}-\textit{HindIII} 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 \textit{guaAB} and \textit{purC}, genetic markers close to \textit{ppk} (20) on the \textit{E. coli} chromosome (data not shown).

\textbf{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 1). 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 \textit{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 \textit{ppk}− strains (Fig. 5); analysis with anti-PPX serum also confirmed...
the absence of PPX (data not shown).

PolyP Content in Wild-type Cells, ppk⁺,ppk⁻ 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/pBC22) 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 Mg²⁺ (see "Materials and Methods"). [³²P]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/10¹⁸ cells in the wild type cells (JM101) and wild-type cells transformed with the vector plasmid (Table I). Another E. coli wild-type strain CF1648 contained 0.44 μg of cellular polyP. Cells overexpressing PPK had polyP at levels over 100-fold
Inorganic Polyphosphate in E. coli

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 (A660 nm) of 1. Cells were harvested by centrifugation and lysed as described (14, 15). PPX activity was measured for the sonicated lysate fraction (14), and PPX activity was determined for the soluble lysate fraction (15). The precipitate 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>
<thead>
<tr>
<th>Strain</th>
<th>PPK Activity (units × 10^−11/g cell)</th>
<th>PPX Activity (units × 10^−11/g cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM101</td>
<td>320</td>
<td>290</td>
</tr>
<tr>
<td>CA10</td>
<td>&lt;1.0</td>
<td>&lt;5.3</td>
</tr>
<tr>
<td>CA11</td>
<td>&lt;2.4</td>
<td>&lt;3.8</td>
</tr>
</tbody>
</table>

Purified PPK (ng) | Culture (μl)
---|---
JM101 | 6 | 18 | 60 | 180 | 50 | 200 | 800
CA10  | 6 | 18 | 60 | 180 | 50 | 200 | 800

Fig. 5. Cellular abundance of PPK. Cells (JM101, CA10) were grown at 37 °C in LB medium to an optical density (OD660 nm) of 1. 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).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>PolyP content (μg/10^11 cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM101 pUC18</td>
<td>Wild type</td>
<td>2.00</td>
</tr>
<tr>
<td>CA10 ppp−</td>
<td>Wild type</td>
<td>2.25</td>
</tr>
<tr>
<td>CA11 ppp−</td>
<td>ppp−</td>
<td>0.16</td>
</tr>
<tr>
<td>JM101 pRC29</td>
<td>ppp−</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Assays of PolyP extracted from different cultures were averaged.

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/10^11 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. 6A), this lag was also observed using a minimal glucose medium (Fig. 6B).

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).

Effects of PolyP Deficiency on Long-term Survival in Stationary-phase, Thermal Resistance, and H2O2 Resistance—E. coli mutant strain CA10 which lacks PPK and PPX exhibited reduced survival in stationary phase (Fig. 7A). 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. 7A). 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
Inorganic Polyphosphate in E. coli

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 overproducers (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. 350–800 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.2 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 rpoS (katF) 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 Ca2+ in the membrane of bacteria competent for DNA transformation (10). The mutant strain with reduced levels of polyP can still acquire competence, al-

---

2 E. Crooke and A. Kornberg, unpublished data.
though with less efficiency than the wild type. 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, 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 pppk gene, phosphate removal from the surrounding medium became far more rapid and complete.6 With a similar plasmid bearing both the pppk 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.

Acknowledgments—We thank Dr. Stanley Cohen (Stanford University) for P1 phage and strain SC364, Dr. Barbara Bachmann (E. coli Genetic Stock Center, Yale University) for strains NX6056 and N3007, Dr. Michael Cashel (National Institutes of Health) for strain CF1648, and Dr. Yuji Kohara (National Institute of Genetics, Japan) for the Kohara phage library.

REFERENCES

E. Crooke, C. E. Castuma, and A. Kornberg, unpublished data.
C. E. Castuma, R. Reusch, and A. Kornberg, unpublished data.
H. Ohtake, personal communication.