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Manipulation of Independent Synthesis and Degradation of Polyphosphate in *Escherichia coli* for Investigation of Phosphate Secretion from the Cell

STEPHEN J. VAN DIEN, SALOMEH KEYHANI, CINDY YANG, AND J. D. KEASLING*

Department of Chemical Engineering, University of California, Berkeley, California 94720-1462

Received 7 October 1996/Accepted 20 February 1997

The genes involved in polyphosphate metabolism in *Escherichia coli* were cloned behind different inducible promoters on separate plasmids. The gene coding for polyphosphate kinase (PPK), the enzyme responsible for polyphosphate synthesis, was placed behind the P_{tac} promoter. Polyphosphatase, a polyphosphate depolymerase, was similarly expressed by using the arabinose-inducible P_{BAD} promoter. The ability of cells containing these constructs to produce active enzymes only when induced was confirmed by polyphosphate extraction, enzyme assays, and RNA analysis. The inducer concentrations giving optimal expression of each enzyme were determined. Experiments were performed in which *ppk* was induced early in growth, overproducing PPK and allowing large amounts of polyphosphate to accumulate (80 μmol in phosphate monomer units per g of dry cell weight). The *ppx* gene was subsequently induced, and polyphosphate was degraded to inorganic phosphate. Approximately half of this polyphosphate was depleted in 210 min. The phosphate released from polyphosphate allowed the growth of phosphate-starved cells and was secreted into the medium, leading to a down-regulation of the phosphate-starvation response. In addition, the steady-state polyphosphate level was precisely controlled by manipulating the degree of *ppx* induction. The polyphosphate content varied from 98 to 12 μmol in phosphate monomer units per g of dry cell weight as the arabinose concentration was increased from 0 to 0.02% by weight.

Many organisms store energy and phosphate in polymers of 3 to more than 1,000 phosphate residues called polyphosphate (17, 33). The diversity of organisms that have been found to contain polyphosphate would indicate a common physiological role; however, little is known about the function of polyphosphate or its effect on other cellular processes.

Recently, there has been great interest in manipulating storage biopolymer levels in microorganisms to investigate how cells survive long periods of nutrient and energy starvation and to produce biodegradable polymers for medical applications. The genes associated with the synthesis and degradation of polyhydroxyalkanoates (9, 23, 27), glycogen (7, 21), and polyphosphate (6, 13) have been cloned and overexpressed in *Escherichia coli*. Polyphosphate kinase (PPK), which catalyzes the reversible transfer of a phosphate group from ATP to a polyphosphate chain, has been purified from *E. coli* (1), and the gene encoding it (*ppk*) has been cloned and overexpressed (3). An exopolyphosphatase (PPX), which irreversibly and processively hydrolyzes phosphate residues from the polyphosphate chain during phosphate starvation, has also been purified (2, 12). The gene encoding polyphosphatase, *ppx*, is located in the same operon as *ppk*.

The manipulation of polyphosphate levels by selective induction of *ppk* and *ppx* allows one to explore the role of these biopolymers during steady-state growth and nutritional deprivation. Since polyphosphate has been implicated in both phosphate and energy storage (18), the controlled synthesis and degradation of polyphosphate could be used in the study of energy metabolism. As the *phoA* promoter has been used extensively for expression of heterologous genes in *E. coli* (26), any effect of polyphosphate on the phosphate starvation (Pho) response could have important consequences for industrial gene expression. Previous studies suggest that cells capable of accessing polyphosphate stores with PPX have reduced alkaline phosphatase expression during starvation for inorganic phosphate (P_i) (25). Control of polyphosphate metabolism would also facilitate the study of its role in heavy metal tolerance (14), providing important applications in the bioremediation of heavy metal contamination in wastewater. However, the independent control of the *ppk* and *ppx* genes by using the native operon has not been possible. Indeed, the independent synthesis and degradation of any storage polymer has not been reported in the literature. This paper describes the construction of a strain with these characteristics, and demonstrates the ability to selectively synthesize and degrade polyphosphate by induction of *ppk* and *ppx*, respectively.

MATERIALS AND METHODS

Strains and plasmids. The *E. coli* strains and plasmids used in this study are listed in Table 1.

Growth medium and conditions. All experiments were conducted in morpholinepropanesulfonic acid (MOPS) minimal medium (20) supplemented with 3% glycerol and 1% Casamino Acids. Glucose was not used as a carbon source because of possible catabolite repression of the arabinose promoter. Induction studies were performed in medium containing 1.32 mM P_i. For experiments in which overproduction of polyphosphate was the objective, the phosphate concentration was increased to 13.2 mM. The medium was supplemented with 3.37 μg of thiamine B1 per ml and 5.0 μg of kanamycin per ml. For experiments involving pSVD5, ampicillin was added to 92 μg/ml. For those involving pSPK1, chloramphenicol was added to 68 μg/ml. The cultures were inoculated with 0.01 volume of stationary-phase culture in identical medium. All experiments were conducted at 37°C in aerated shake flasks of at least five times the liquid volume.

PCR. Primers for PCR consisted of a 19- to 23-bp region complementary to the DNA template, followed by 5 bp coding for a strong ribosome binding site (GGAGG), and 14 to 19 bp containing the desired restriction site and a nonspecific region to facilitate enzyme binding. The PCR mixture (100 μl) consisted of PCR buffer (Perkin-Elmer, Foster City, Calif.), 0.1 ng of DNA template (pBC9) per ml, 0.50 pmol each of 3′ and 5′ primers per ml, 200 μM each deoxynucleoside triphosphate, and 2 U of Perkin-Elmer *Taq* polymerase. A PTC 1000 programmable thermocycler (M. J. Research Labs Inc., Watertown, Mass.)

* Corresponding author. Phone: (510) 642-4862. Fax: (510) 642-4778. E-mail: keasling@garnet.berkeley.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Reference or source
E. coli K-12		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ-*thi-1 gyrA96 relA1*	Life Technologies, Inc., Gaithersburg, Md.
CA38[a]	Δ(*lac-proAB*) *supE thi srl-300*::Tn*10 recA56* F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*) *ppk*::*kan*	6
JS4	F$^-$ *araD139* Δ(*ara leu*)*7697*, Δ(*lac*)$_{X74}$ *galU galK hsdR2*(r_K^- m_K^+) *mcrA mcrBC rpsL* (Str^r) *thi recA1*	Bio-Rad Laboratories, Inc., Richmond, Calif.
Plasmids		
pUC18	Amp^r, P_{lac}	Promega
pBC9[b]	pUC18, *ppk-ppx*	2
pSK14[c]	pUC18, P_{tac}::*ppk*	This study
pSK16[c]	pUC18; P_{tac}::*ppx*	This study
pMMB206	Cm^r, *lacI*^{q}-P_{tac}, medium-copy-number plasmid	19
pSPK1	pMMB206, P_{tac}::*ppk*	This study
pBAD18	Amp^r, *araC*-P_{BAD}, high-copy-number plasmid	11
pSVD5	pBAD18, P_{BAD}::*ppx*	This study
pKLJ09	pBAD18, P_{BAD}::*lacZ*	Gift from K. L. Jones, U. C. Berkeley

[a] CA38 has a kanamycin resistance (Kan^r) gene inserted into the chromosomal *ppk* gene that inactivates both the *ppk* and *ppx* genes.
[b] pBC9 contains the *ppk* and *ppx* genes under control of their native promoters (2).
[c] *ppk* and *ppx* genes are the cloned PCR products with highest activity.

was used to repeat the following cycle 15 times: melt for 1 min at 94°C, anneal at 55°C for 2 min, and extend at 72°C for 3 min. The *ppk* and *ppx* products were then cloned into pUC18 behind the P_{lac} promoter by using the restriction sites created by the PCR procedure (*Pst*I at the start of each gene and *Hin*dIII at the end), and transformed into DH5α. All the restriction enzymes used were obtained from Boehringer Mannheim (Indianapolis, Ind.).

Cell lysis and enzyme assays. Culture samples of 25 ml were taken at the indicated times, harvested by centrifugation at 12,000 × *g* for 10 min, and resuspended in 50 mM Tris (pH 7.5)–10% sucrose to give a final optical density at 590 nm (OD_{590}) of 80. The samples were frozen in liquid nitrogen and stored at −87°C prior to lysis. They were thawed on ice, and 75 μl was combined with 75 μl of lysis buffer containing 50 mM Tris (pH 7.5), 10% sucrose, 300 mM NaCl, 90 mM EDTA, and 3 mg of lysozyme per ml. The resulting mixture was incubated on ice for 1 h. Lysis was improved by repeated freeze-thawing cycles alternating between liquid nitrogen and 37°C followed by sonication twice for 8 s at 4°C with a Branson Sonifier (Branson Ultrasonics Corp., Danbury, Conn.) fitted with a microtip. The samples were then centrifuged at 10,000 × *g* for 30 min, and the supernatant was frozen in liquid nitrogen and stored at −87°C prior to the assays. Dilutions of the samples were made in 50 mM HEPES-KOH (pH 7.5)–10% sucrose–5 mM $MgCl_2$–40 mM $(NH_4)_2SO_4$. The total protein concentration was measured by the method of Bradford (Bio-Rad, Richmond, Calif.) (5). The PPK and PPX assays were performed by the methods described previously by Sharfstein and Keasling (24). Alkaline phosphatase activity was determined by measuring the rate of liberation of *p*-nitrophenol from *p*-nitrophenol phosphate (4).

mRNA analysis. RNA probes for *ppk* and *ppx* were made with fragments from pBC9 as templates. The 1,184-bp *Hin*cII and 390-bp *Kpn*I digestion fragments were used for *ppk* and *ppx*, respectively. The gene fragments were cloned behind the bacteriophage T7 promoter on pGEM-4Z (Promega, Madison, Wis.). Only clones in which the gene was inserted in the reverse direction were chosen, so that transcription would produce antisense RNA. Radiolabelled probes were created by in vitro transcription with the Promega Riboprobe kit and [α-^{32}P]CTP.

For total-RNA isolation, 200 μl of the 50 mM Tris (pH 7.5)–10% sucrose cell resuspensions described above was used. RNA was isolated with the Totally RNA kit (Ambion, Austin, Tex.). To eliminate potential DNA contamination, each sample was incubated with 4 U of RNase-free DNase (Promega) for 20 min at 37°C. The samples were then extracted with an equal volume of phenol-chloroform (1:1), precipitated with 2 volumes of 95% ethanol, and resuspended in 50 μl of hybridization buffer (80% formamide, 40 mM MOPS [pH 7.0], 400 mM NaCl, 1 mM EDTA). The RNA concentrations were measured spectrophotometrically at 260 nm (22); they generally ranged from 1 to 3 μg/μl.

Transcripts of *ppk* and *ppx* were detected by the S1 nuclease assay in a procedure similar to that described by Farrell (8). For each sample, 6 μg of total RNA and approximately 10^6 cpm of probe were added to 20 ml of hybridization buffer. The samples were denatured by heating to 85°C for 10 min and immediately transferred to the hybridization temperature of 60°C for 3 h. To each tube was added 160 μl of water, 20 μl of 10× S1 digestion buffer (50% glycerol, 1 M NaCl, 300 mM sodium acetate [pH 4.6], 20 mM $ZnSO_4$), and 200 U of S1 nuclease (Promega). Digestion was carried out for 2.5 h at 37°C and stopped by extraction with an equal volume of phenol-chloroform (1:1). The RNA was precipitated by addition of 40 μl of 5 M ammonium acetate, 40 μg of tRNA (Sigma, St. Louis, Mo.), and 400 μl of 95% ethanol for 2 h at −20°C and recovered by centrifugation at 10,000 × *g* for 30 min. The pellets were allowed to dry for approximately 30 min and then resuspended in 10 μl water plus 2 μl of 6× loading buffer (50% glycerol, 1 mM EDTA, 2.5 mg of xylene cyanol FF per ml, 2.5 mg of bromophenol blue per ml). The samples were run on an agarose gel (1.5% agarose for *ppk*, 2.2% for *ppx*), which was then air dried under vacuum at room temperature for 30 min and exposed to X-ray film (Kodak, Rochester, N.Y.) for 8 to 14 h at −87°C. The image was scanned on a flat-bed scanner, and the density of the bands was quantified with the NIH Image program. To eliminate RNase contamination, all reagents used in the RNA analysis were pretreated with 0.1% diethylpyrocarbonate for 8 h at 37°C, and then autoclaved at 121°C for 1 h. Positive and negative controls for this analysis were performed with unlabelled antisense and sense probes, respectively, in place of the RNA samples. An additional negative control to demonstrate the specificity of the probe was performed with a total-RNA sample from CA38 pUC18, for which no signal was detected.

Polyphosphate analysis. Samples of 25 ml were taken for polyphosphate extraction (25). Polyphosphate was then identified by ^{31}P nuclear magnetic resonance spectroscopy as a peak with a chemical shift of −23 ppm. The peak area was integrated relative to that of the internal standard methylenediphosphonic acid, and the polyphosphate was quantified by comparing this area to that of a standard sample in which the polyphosphate was hydrolyzed to P_i. The P_i was subsequently analyzed with diagnostic kit 360-UV (Sigma, St. Louis, Mo.).

Phosphate secretion from the cell. To determine if phosphate generated during the degradation of polyphosphate can be secreted from the cell, [^{32}P]polyphosphate was synthesized by growing CA38 pSPK1 pSVD5 in the presence of 0.5 μCi of [^{32}P]H_3PO_4 (Amersham Life Science, Arlington Heights, Ill.) per ml and 0.134 mM isopropyl-β-D-thiogalactopyranoside (IPTG). When the culture had grown to an OD_{590} of 1.4, it was centrifuged briefly at 12,000 × *g* and resuspended in unlabelled medium. The culture was centrifuged again after 20 min and resuspended in phosphate-free medium. It was then separated into two test tubes, which were sealed with rubber septa. Arabinose was added to one of the tubes to 0.02% (by weight) to induce *ppx*. Samples of 1 ml were taken at the indicated times, and the cells were separated from the medium by centrifugation. The radioactivity in 0.9 ml of the supernatant was then counted by liquid scintillation and converted to appropriate units based on the specific activity of the original culture.

RESULTS

Construction of plasmids for controlled *ppk* and *ppx* expression. PCR was used to introduce restriction sites around the *ppk* and *ppx* genes for convenient cloning into pUC18. The DH5α transformants were grown in MOPS medium to an OD_{590} of 0.5 and induced by the addition of IPTG to 0.134 mM. The cultures were harvested and assayed for PPK and PPX activities, and the clone with the highest activity of each

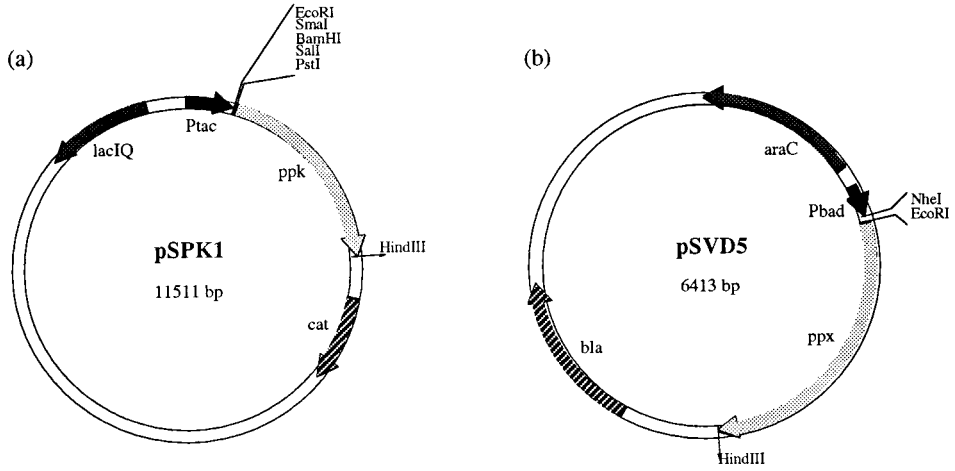

FIG. 1. Plasmid constructs. (a) pSK14 was digested with *Pst*I and *Hin*dIII, and the 2,200-bp fragment containing the *ppk* PCR product was subcloned into pMMB206, creating pSPK1. Ptac, *trp-lac* hybrid promoter; *lacIQ*, *lac* repressor gene; bla, Amp^r gene; ppk, PCR product of *ppk* gene giving the highest activity of all clones screened. (b) pSPK16 was digested with *Eco*RI and *Hin*dIII, and the 1,800-bp fragment containing the *ppx* PCR product was subcloned into pBAD18, creating pSVD5. Pbad, arabinose promoter; araC, arabinose repressor gene; cat, Cam^r gene; ppx, PCR product of the *ppx* gene giving the highest activity of all clones screened.

was selected: pSK14 (*ppk*) and pSK16 (*ppx*). It should be emphasized that these clones are not necessarily exact copies of the *E. coli* genes; errors may have been introduced during PCR. For the purpose of selectively synthesizing and degrading polyphosphate, these possible errors are not important.

The *ppk* gene from pSK14 was subcloned into pMMB206 (19) behind the P_{tac} promoter to create pSPK1 (Fig. 1a). This plasmid contains the Cam^r gene, as well as the repressor gene $lacI^q$ to ensure low basal *ppk* expression. The *ppx* gene was subcloned from pSK16 into pBAD18 (11) behind the arabinose P_{BAD} promoter to create pSVD5 (Fig. 1b). This plasmid is Amp^r and contains the *araC* repressor. These plasmids were transformed into CA38, both separately and together. Since they have different origins of replication, both plasmids are stable in the same cell.

Induction of *ppk*. CA38 (pSPK1) was grown, as described in Materials and Methods, to an OD_{590} within the range 0.5 to 0.6. The *ppk* gene was then induced by the addition of IPTG to different concentrations in each of five flasks: 0, 0.0335, 0.067, 0.134, and 0.268 mM. When the cultures had reached an OD_{590} of approximately 1.25 (90 min after induction), samples were taken from each flask for enzyme and RNA analysis. Additional samples were taken 200 min after induction, when the cells were in the stationary phase at an OD_{590} of 2.5. PPK assays and *ppk* mRNA analysis demonstrated that optimal induction (310 U of PPK activity/μg of protein) was achieved with 0.134 mM IPTG (Fig. 2). For comparison, the PPK activity in an exponentially growing wild-type *E. coli* cell is approximately 5 U/μg (24). The uninduced samples had very low transcript and enzyme activity levels, indicating that the repressor gene $lacI^q$ maintained tight control over the operon.

To confirm that induced cells were producing polyphosphate, cultures of CA38 (pSPK1) were grown and induced, when the OD_{590} was approximately 0.32, with IPTG to 0, 0.067, and 0.201 mM IPTG. Samples were taken 90 and 185 min after induction for polyphosphate isolation. The polyphosphate peaks were integrated relative to the internal standard and quantified by comparison to a known sample. Polyphosphate accumulated to approximately 93 μmol in phosphate monomer units (P_i) per g of dry cell weight (DCW) (Table 2).

Induction of *ppx*. CA38(pSVD5) was grown as described in Materials and Methods to an OD_{590} within the range 0.35 to 0.45. The *ppx* gene was induced by the addition of arabinose to different concentrations in each of five flasks: 0, 0.0005, 0.002, 0.02, and 0.2% (by weight). When the cultures had reached an OD_{590} of approximately 0.60 (70 min after induction), samples were taken from each flask for enzyme and RNA analysis. Samples were also taken 140 min after induction, when the cells were at an OD_{590} of 0.8. PPX assays and *ppx* mRNA analysis demonstrated that optimal induction (228 U of PPX activity/μg of protein) was achieved with 0.02% arabinose (Fig. 3). For comparison, the PPX activity in an exponentially growing wild-type *E. coli* cell is approximately 3.3 U/μg (24). The uninduced samples had very low transcript and activity levels, indicating that the repressor gene *araC* maintained tight control over the operon.

Polyphosphate synthesis and degradation. Two cultures of JS4(pSPK1 pSVD5) were grown in medium containing 0.134

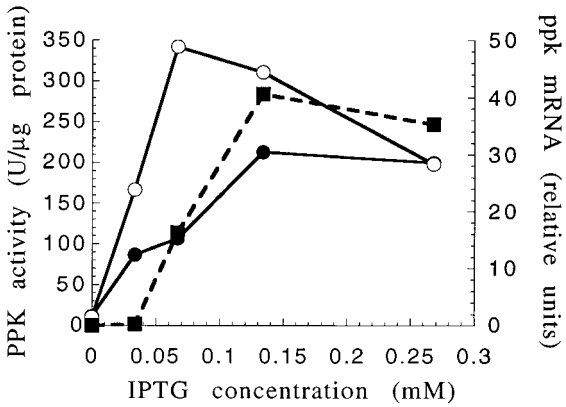

FIG. 2. Induction of *ppk* in CA38 (pSPK1) by addition of IPTG. Solid circles, PPK activity as a function of IPTG concentration in samples taken 90 min after induction; open circles, PPK activity in samples taken 200 min after induction (PPK activities are expressed as units per microgram of total protein in the cell extract; 1 unit of activity is defined as the amount of enzyme needed to incorporate 1 pmol of phosphate into polyphosphate in 1 min at 37°C); solid squares, *ppk* mRNA levels as a function of IPTG concentration 90 min after induction, as determined by the S1 nuclease assay with an RNA probe complementary to an 1,184-nucleotide segment of the *ppk* transcript.

TABLE 2. Polyphosphate levels as a function of inducer concentration in CA38(pSPK1)[a]

IPTG Concn (mM)	Amt of polyphosphate (μmol of P_i/g DCW)[b] after:	
	90 min	185 min
0	0	0
0.067	4.7 ± 0.8	24.4 ± 0.7
0.201	34.4 ± 3.0	92.8 ± 19.1

[a] Polyphosphate was quantified by integrating the peak at −23 ppm relative to that of the internal standard (MDPA) at 16 ppm. The peak area was converted to phosphate content by comparison to a sample in which the polyphosphate was subsequently hydrolyzed and assayed for P_i.

[b] The sample times given are the times after IPTG induction. Each datum point represents three independent experiments, and results are tabulated as mean ± standard deviation. No polyphosphate was detected in any of the cultures with no IPTG added.

mM IPTG, so that the cells would be at steady state with respect to polyphosphate production at the time when the samples were taken. When the OD_{590} of the cultures reached 1.0, one culture was induced with arabinose to a final concentration of 0.02% by weight. Samples were taken periodically for polyphosphate extraction both before and after induction. The polyphosphate content of the uninduced culture remained constant throughout the experiment, while that of the induced culture began decreasing once arabinose was added. The results of a representative experiment are shown in Fig. 4a. Although the absolute amount of polyphosphate in the cells varied by as much as 20% from one experiment to the next, the difference in the polyphosphate levels between cultures that were not induced with arabinose and those that were induced varied significantly less. The differences in the polyphosphate levels from three independent experiments are included in Fig. 4b.

To ensure that the cultures remained in exponential phase for the samples at 1,200 min, they were back-diluted into identical medium. Since the cells were allowed to grow for many generations since induction, these data points represent the steady-state polyphosphate levels. To ensure that the decrease in polyphosphate content was specifically due to PPX, rather than the metabolic burden of overproducing protein, the steady-state experiment was repeated with JS4(pSPK1 pKLJ09). This strain produced β-galactosidase, rather than PPX, when induced with arabinose. The polyphosphate concentrations for these cultures with 0 and 0.02% arabinose were 86 and 81 μmol of P_i/g DCW, respectively, which were very similar to those in the uninduced JS4(pSPK1 pSVD5) (Fig. 4a).

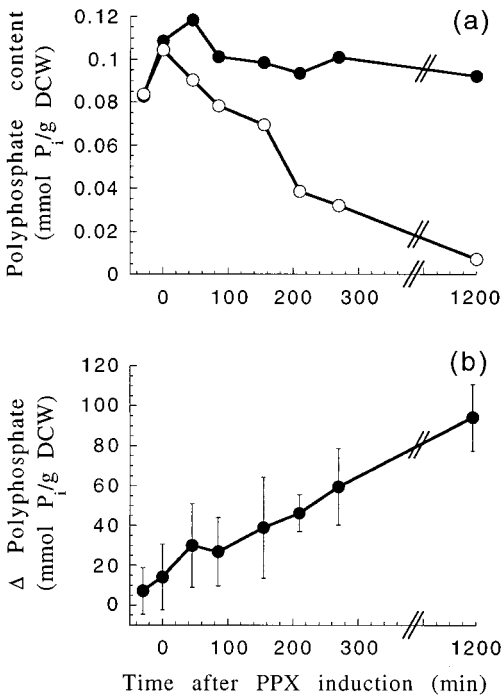

FIG. 4. Polyphosphate synthesis and degradation in JS4(pSPK1 pSVD5). Arabinose was added at time zero. Polyphosphate concentrations were measured and reported as in Table 1. (a) Data from a representative experiment. Solid circles, no arabinose added; open circles, arabinose added to 0.02% by weight. Datum points at 1,200 min represent steady-state polyphosphate levels, as described in the text. (b) Difference between polyphosphate concentration in a culture with no arabinose added and one in which arabinose was added to 0.02% by weight at time zero. Each datum point represents the mean of three independent experiments, with the error bars indicating the standard deviation.

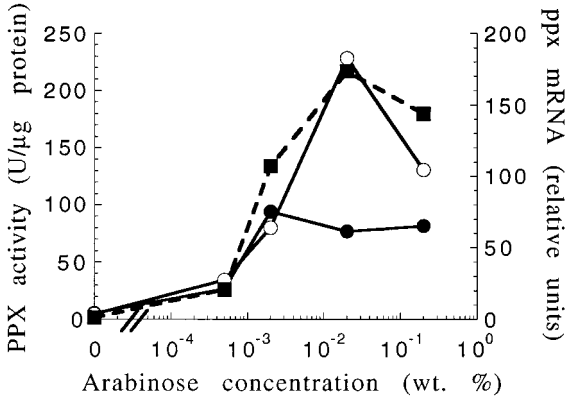

FIG. 3. Induction of *ppx* in CA38(pSVD5) by addition of arabinose. Solid circles, PPX activity as a function of arabinose concentration in samples taken 70 min after induction; open circles, PPX activity in samples taken 140 min after induction (PPX activities are expressed as units per microgram of total protein in the cell extract; 1 unit of activity is defined as the amount of enzyme needed to liberate 1 pmol of phosphate from polyphosphate in 1 min at 37°C); solid squares, *ppx* mRNA levels as a function of arabinose concentration 70 min after induction, as determined by the S1 nuclease assay with an RNA probe complementary to a 390-nucleotide segment of the *ppx* transcript. Although points at 0% arabinose cannot be displayed on a log scale, these data are included to show basal expression levels.

Manipulation of steady-state polyphosphate levels. In an independent experiment, the method described above was used to measure the steady-state polyphosphate content as a function of arabinose concentration (Fig. 5). Arabinose concentrations between 0 and 0.02% resulted in intermediate levels of polyphosphate.

Phosphate secretion. To determine whether P_i generated during polyphosphate degradation by PPX can be released from the cell, [^{32}P]polyphosphate was synthesized in vivo in CA38(pSPK1 pSVD5) by inducing the *ppk* gene only. The culture was then divided in half and shifted to anaerobic phosphate-free conditions, and *ppx* was induced with 0.02% arabinose. During a period of 230 min following the induction of *ppx*, 48 μmol of P_i per g DCW was secreted into the medium (Fig. 6). As a control, the same experiment was conducted with CA38, which contains no polyphosphate. The concentration of P_i in the medium with arabinose added to a culture containing polyphosphate was threefold greater than that when arabinose was not added or when the culture did not contain polyphos-

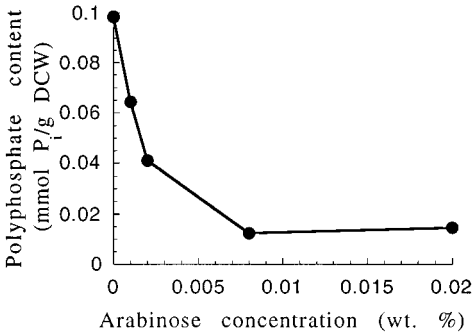

FIG. 5. Steady-state polyphosphate level as a function of arabinose concentration in JS4(pSPK1 pSVD5), measured and reported as in Table 1.

phate. This experiment has been repeated with other strains containing plasmids pSPK1 and pSVD5, each time yielding the same qualitative result (data not shown). Due to differences in the steady-state polyphosphate concentration among strains, the numerical values obtained from the different experiments cannot be directly compared.

A similar experiment was performed with unlabeled medium to measure cell growth and alkaline phosphatase activity during P_i starvation with and without *ppx* induction. The culture that received 0.02% arabinose at the time of the shift continued to grow for approximately 130 min, whereas cell growth was strongly inhibited in the culture without arabinose (Fig. 7a). Alkaline phosphatase activity, which is an indicator of the phosphate starvation response, was reduced by approximately 50% in the culture receiving arabinose (Fig. 7b).

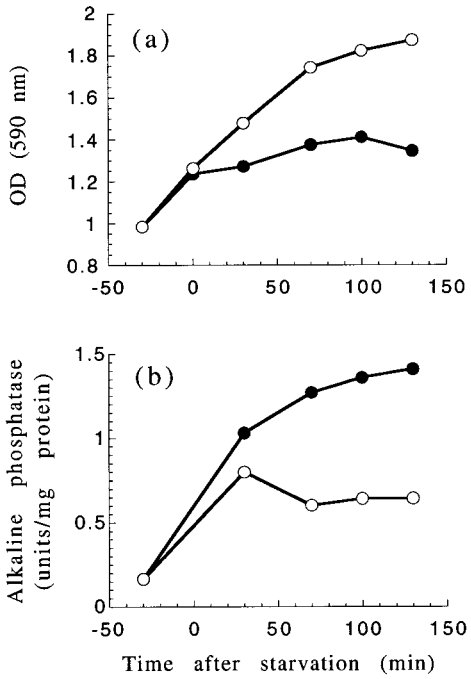

FIG. 7. Cell growth and alkaline phosphatase production upon a shift to phosphate-free conditions. (a) Cell growth (OD_{590}). (b) Alkaline phosphatase activity. One unit of activity is defined as the amount of enzyme needed to release 1 mmol of P_i per min. Solid circles, CA38(pSPK1 pSVD5), no arabinose added; open circles: CA38(pSPK1 pSVD5), arabinose added to 0.02% by weight at the time of the shift.

DISCUSSION

This paper describes the construction of an *E. coli* strain in which the genes responsible for the synthesis and degradation of polyphosphate, *ppk* and *ppx*, were placed under the control of separate, inducible promoters. The expression vectors chosen for this purpose had different origins of replication and antibiotic resistances, so that they would be stable in the same cell. Since P_{BAD} is the weaker of the two promoters used, it was desirable to use a high-copy-number plasmid for expression with P_{BAD}. P_{BAD} also has extremely tight regulation (11), and so it was used for PPX expression. Since PPK is expressed early in growth during the polyphosphate synthesis and degradation experiments, uninduced PPK activity would not present much of a problem. However, the induction studies demonstrate low basal activity with both promoters (Fig. 2 and 3). When induced with IPTG, polyphosphate can accumulate to levels in 100-fold excess of those seen in wild-type strains. This polyphosphate can then be degraded to P_i by PPX when induced with arabinose.

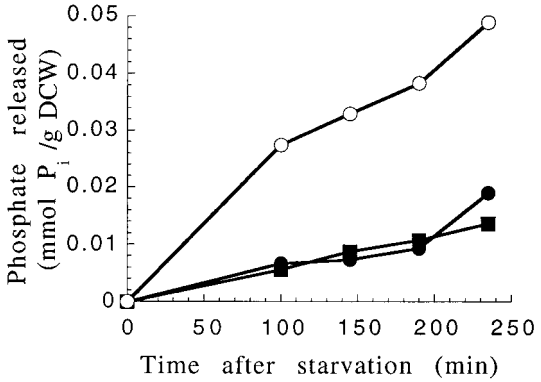

FIG. 6. Phosphate release into medium upon shift to phosphate-free, anaerobic conditions. Appearance of the ^{32}P label in the culture supernatant was measured and converted to appropriate units based on the specific activity of the original culture. Solid circles, CA38(pSPK1 pSVD5), no arabinose added; open circles, CA38(pSPK1 pSVD5), arabinose added to 0.02% by weight at the time of the shift; solid squares, CA38, arabinose added to 0.02% by weight at the time of the shift.

For the degradation of polyphosphate in vivo by using PPX produced by arabinose induction, it was desirable to use a strain that cannot metabolize arabinose. First, it was critical to show that the change in polyphosphate content cannot be attributed to the utilization of a different carbon source. Second, the use of such a strain ensured that the arabinose added for induction was never depleted. The arabinose mutant chosen for this study was JS4. It is not important that JS4 has functional *ppk* and *ppx* genes on the chromosome, since the activity levels due to chromosomal expression were very low. For example, the basal PPX activity of CA38 (pSPK1 pSVD5) was 0.97 U/μg of protein, while that of JS4 (pSPK1 pSVD5) was 2.27 U/μg of protein. In addition, the induction of *ppk* and *ppx* in JS4(pSPK1 pSVD5) with 0.134 mM IPTG and 0.02% arabinose resulted in PPK and PPX activity levels comparable to those seen in CA38(pSPK1) and CA38(pSVD5).

The synthesis and degradation of polyphosphate can be represented by the reaction pathway in Fig. 8 (2, 15, 16). When the cells are in balanced growth, the polyphosphate concentration should be constant, such that:

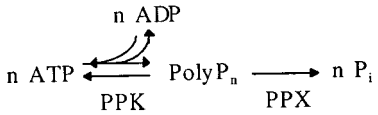

FIG. 8. Pathway for the synthesis and degradation of polyphosphate ($PolyP_n$).

$$\frac{d[\text{polyP}]}{dt} = 0 = k_1[\text{ATP}] - k_{-1}[\text{polyP}][\text{ADP}] - k_2[\text{polyP}]$$

$$[\text{polyP}] = \frac{k_1}{k_{-1}[\text{ADP}] + k_2}[\text{ATP}]$$

where k_1 and k_{-1} are pseudo-first-order rate constants for the forward and reverse PPK reaction and k_2 is the rate constant for the PPX reaction. As long as the energy charge is constant, the polyphosphate level in the cells will remain relatively constant. When PPX is present to degrade polyphosphate reserves, this level will be lower than when PPX is absent ($k_2 = 0$). This behavior is observed in Fig. 4 after *ppx* induction with arabinose. In addition, the polyphosphate content in the cells can be manipulated by changing the arabinose concentration, thus altering the flux through the degradation pathway (Fig. 5). Although PPK activities should be similar in all three flasks, it is conceivable that the metabolic burden of overproducing PPX reduces the amount of ATP available to synthesize polyphosphate. This possibility was eliminated by using strain JS4(pSPK1 pKLJ09) as a control. When induced with arabinose, it should have a similar burden to that of overexpression from JS4(pSPK1 pSVD5) but without functional PPX being produced. Since there is no decrease in the polyphosphate level when this strain is induced with arabinose, the phenomenon observed in Fig. 4 and 5 is due to PPX rather than a difference in the rate of polyphosphate synthesis.

The application of these constructs to the investigation of bacterial phosphate transport has been demonstrated here. The release of phosphate from the cells observed in Fig. 6 is consistent with the observation that the internal P_i concentration in *E. coli* will not rise above 30 mM (34). Given the polyphosphate levels prior to *ppx* induction, the intracellular P_i concentration resulting from the polyphosphate degradation seen in Fig. 4 should be over 100 mM. The shift to phosphate-free medium was made to create a favorable P_i gradient for release, and the shift to anaerobic conditions was intended to slow growth significantly enough that the amount of P_i produced by polyphosphate hydrolysis would be in excess of that required for growth. In addition, phosphate release in *Acinetobacter* (28) has been observed only with a shift to anaerobic growth. Since precise control over when polyphosphate degradation occurs was an absolute requirement for this experiment, these results could not have been obtained with previously engineered strains in which *ppk* and *ppx* were under the control of the same promoter (2).

The results seen in Fig. 7 confirm the previous observation that the Pho response is affected by the ability of cells to access polyphosphate reserves with PPX (25). A model in which the P_i liberated from polyphosphate by PPX is both used for cell growth and transported out of the cell can now be proposed. The resulting increased level of P_i in the periplasmic space down-regulates the Pho response.

The constructs developed and tested here should prove useful in future studies of bacterial phosphate transport and energy metabolism. The ability of cells to export phosphate through the phosphate inorganic-transport (Pit) system is well documented for *Acinetobacter* strains (28–30) and has been demonstrated in *E. coli* proteoliposomes (31). A continuation of this work could involve elucidation of the mechanism for phosphate transport out of the cell. The ability of cells to use polyphosphate to regenerate ATP from ADP under energy-limited conditions can also be investigated with pSPK1, the plasmid containing the inducible *ppk*. A *Pseudomonas* species that contains polyphosphate has been shown to maintain a high adenylate energy charge during carbon starvation, while that of wild-type *E. coli* drops drastically (10, 32).

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