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Genetic Manipulation of Polyphosphate Metabolism Affects Cadmium Tolerance in *Escherichia coli*

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The polyphosphate metabolic pathways in *Escherichia coli* were genetically manipulated to test the effect of polyphosphate on tolerance to cadmium. A polyphosphate kinase (*ppk*) and polyphosphatase (*ppx*) mutant strain produced no polyphosphate, whereas the same strain carrying multiple copies of *ppk* on a high-copy plasmid produced significant quantities. The doubling times of both strains increased with increasing cadmium concentrations. In contrast, the mutant strain carrying multiple copies of *ppk* and *ppx* produced 1/20 of the polyphosphate found in the strain carrying multiple copies of *ppk* only and showed no significant increase in doubling time over the same cadmium concentration range.

Microorganisms have evolved a number of heavy metal tolerance mechanisms: exclusion, excretion, sequestration, and transformation (13, 19, 29). These mechanisms are primarily active, in that they are induced in the presence of heavy metals. Microorganisms also have passive tolerance mechanisms that are not induced in the presence of heavy metals but nonetheless improve cellular tolerance to metals. It has been suggested that one such mechanism is intracellular chelation of heavy metals by long-chain anionic polymers of phosphate called polyphosphate (polyP) (16, 23). Although Ca^{2+}, K^+, and Mg^{2+} are most often associated with polyP bodies, heavy metals have been found in the polyP granules of certain bacteria (5, 6, 15, 20, 22), in the polyP-containing vacuoles of *Saccharomyces cerevisiae* (8, 11, 14, 18, 28), and in the polyP of algae (16). Rachlin and coworkers have proposed that cells use polyP to detoxify heavy metals once they enter the cell (17). This is supported by the work of Sicko-Goad and Lazinsky (22), who found that as the number of polyP bodies decreased lead, caused increasing cytological changes to the point of cell death; by the work of Pettersson and coworkers (15), who found that *Anabaena cylindrica* accumulates significantly more aluminum during growth on high-phosphate medium (when polyP bodies are formed) than during growth on low-phosphate medium; and by the work of Aiking and coworkers (2), who found that *Klebsiella aerogenes* accumulates P_i to detoxify cadmium. These reports indicate that the amount of polyP in a cell correlates with cellular tolerance to heavy metals.

Several laboratories have investigated the correlation between the polyP level and resistance to heavy metals by varying the supply of phosphate in the medium and thereby manipulating the intracellular polyP level (9). Unfortunately, excess extracellular phosphate can lead to precipitation of metal-phosphate complexes in the medium, whereas limitation of phosphate can lead to phosphate starvation of the cell. Therefore, any result obtained with these techniques is questionable.

To circumvent these problems, we genetically manipulated the polyP level in *Escherichia coli* and examined the effect of the resulting polyP level on cell growth during exposure to heavy metals. Two enzymes regulate the polyP level in *E. coli*: polyP kinase (PPK) reversibly synthesizes polyP from ATP (1), and exopolyPase (PPX) irreversibly hydrolyzes polyP to form P_i (4). The genes for both enzymes have been cloned and overexpressed (3, 4). The intracellular polyP level was manipulated by transforming a *ppk ppx* mutant strain with a high-copy plasmid carrying either the *ppk* gene or the *ppk* and *ppx* genes. The resulting strains were tested for PPK and PPX activities, for polyP level, and for growth in the presence of cadmium.

The bacterial strain used was *E. coli* K-12 CA38 [Δ(*lac-proAB*) *supE thi srl-300*::Tn*10 recA56* F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*) *ppk*::*kan*]. This strain has a kanamycin resistance gene inserted into the *ppk* gene that inactivates expression of both *ppk* and *ppx*. This strain was transformed with three different plasmids. Plasmid pUC18 is a commercial cloning vector onto which the genes for the polyP pathways were cloned and was the control plasmid for the experiments. Plasmid pBC9 contains the entire polyP operon—two promoters, *ppk*, and *ppx* (3, 4). Plasmid pBC29 contains only the promoters and *ppk*. Both plasmids are gifts from Arthur Kornberg. The strains were grown in minimal medium buffered with 2-(*N*-morpholino)propanesulfonic acid (MOPS) (12). The medium was supplemented with glucose (4 g/liter), ampicillin (25 mg/liter), and thiamine (3.37 mg/liter).

Enzyme activities. The PPK and PPX enzyme activities were assayed as described by Sharfstein and Keasling (21). Approximately 250 ml of cells was centrifuged at 10,000 × *g*. The pellet was resuspended in 1 ml of a solution of 50 mM Tris-HCl (pH 7.5) and 10% sucrose and then frozen in liquid nitrogen. The samples were stored at −87°C prior to lysis. The cell samples were thawed on ice and then recentrifuged at 10,000 × *g*. The cell pellet was resuspended in 0.5 ml of a solution of 50 mM Tris-HCl (pH 7.5) and 10% sucrose. Approximately half (0.5 ml) of the resuspended cells were combined with 0.25 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10% sucrose, 300 mM NaCl, 90 mM EDTA, and 3 mg of lysozyme per ml. The mixture was incubated on ice for 1 h. The lysis was improved by repeated freeze-thawing cycles in liquid nitrogen and a 37°C water bath. The sample was incubated on ice for an additional hour, sonicated for 30 s at 4°C, and centrifuged at 10,000 × *g* for 10 min. Assays were performed on the supernatant.

The PPK assay measured the production of acid-insoluble [^{32}P]polyP. The reaction mixture contained 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–KOH (pH 7.2), 40 mM $(NH_4)_2SO_4$, 4 mM $Mg(CH_3CO_2)_2$, 66.7 mM phosphocreatine, 100 μg of creatine kinase, 1 mM ATP, 300

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TABLE 1. PolyP levels and PPK and PPX activities of strains used in this study[a]

Strain	*ppk* gene	*ppx* gene	PPK sp act	PPX sp act	Amt of PolyP (μmol of P_i/OD_{600} unit)[b]
E. coli CA38/pUC18	−	−	ND[c]	21	ND
E. coli CA38/pBC29	+	−	1,041	23	5.25
E. coli CA38/pBC9	+	+	1,085	290	0.23

[a] PPK and PPX activities were measured by using the assays described in the text. One unit of PPK activity is equal to 1 pmol of P_i incorporated into polyP per min at 37°C. One unit of PPX activity is equal to 1 pmol of P_i liberated from polyP per min at 37°C. Specific activity is reported as units of enzyme activity per microgram of total cell protein. The measurable polyPase activity in CA38/pUC18 and CA38/pBC29 is due to the presence of an intracellular polyPase other than PPX that is expressed at low levels (10). PolyP levels were quantified by extracting the polyP from 750 ml of cells, hydrolyzing the extracted polyP in 1 N HCl at 95°C for 15 min, and measuring the amount of P_i.

[b] OD_{600}, optical density at 600 nm.

[c] ND, not detectable.

μCi of [γ-^{32}P]ATP (10 mCi/mmol), and PPK in a volume of 250 μl. The mixture was incubated at 37°C for 15 min. The reaction was stopped by addition of 250 μl of 7% $HClO_4$ and 50 μl of bovine serum albumin. [^{32}P]polyP was collected on glass filters and washed with 8 ml of a solution of 1 M HCl and 0.1 M PP_i. The amount of label incorporated into polyP was measured by liquid scintillation counting. One unit of activity is equal to 1 pmol of P_i incorporated into polyP per min.

The PPX assay measured the formation of P_i by hydrolysis of polyP. The reaction contained 25 mM HEPES-KOH (pH 8.0), 0.5 mM $Mg(CH_3CO_2)_2$, 0.5 mM dithiothreitol, 66.7 mM $NH_4CH_3CO_2$, 66.7 μM [^{32}P]polyP (in P_i residues) in a final volume of 15 μl. (The [^{32}P]polyP was made with purified PPK and [γ-^{32}P]ATP [10].) The mixture was incubated for 30 min at 37°C. A 1-μl sample of the reaction mixture was spotted onto a polyethyleneimine thin-layer chromatography plate and developed with 0.4 M LiCl and 1 M HCOOH. ADP and ATP (12.5 nmol of each) were spotted at the origin as markers for the separation. The plates were exposed to UV light to visualize the ATP and ADP spots, cut into two strips according to the markers (i.e., origin, ATP, and ADP), and counted by using liquid scintillation. One unit of activity is equal to 1 pmol of phosphate liberated from polyP per min.

The results of the enzyme assays are listed in Table 1. The data indicate that the strains carrying the *ppk* gene on a high-copy plasmid (CA38/pBC29 and CA38/pBC9) had higher PPK activities than the strain carrying the control plasmid (CA38/pUC18). The strain carrying both the *ppk* and *ppx* genes on a high-copy plasmid (CA38/pBC9) had higher PPX activity than either CA38/pUC18 or CA38/pBC29.

PolyP levels in the engineered strains. PolyP was isolated from 750 ml of culture and analyzed by ^{31}P nuclear magnetic resonance spectroscopy as described by Sharfstein and Keasling (21). The ^{31}P nuclear magnetic resonance spectra were collected on a Bruker AM-400 spectrometer at a frequency of 161.98 MHz. The sweep width was set to 31,250 Hz. The spectra were acquired with a 6.0-μs pulse (60° pulse angle) with an acquisition time of 1.04 s and with no recycle delay. Because a fast recycle rate was used, the spectra are not rigorously quantitative. However, since all spectra were obtained under the same conditions, comparisons can be made between different spectra. Spectra were processed by using exponential multiplication with 5-Hz line broadening. Typical spectra from the genetically engineered strains are shown in Fig. 1.

Since the ^{31}P nuclear magnetic resonance spectra were not rigorously quantitative, the amount of polyP in terms of P_i residues was measured by hydrolyzing the polyP and determining the amount of P_i by reaction with ammonium molybdate in the presence of sulfuric acid (diagnostic kit 360-UV; Sigma Chemical Company, St. Louis, Mo.) (7). The amounts of polyP in the strains are also listed in Table 1. It should be noted that *E. coli* CA38/pUC18, which had no functioning *ppk* or *ppx* gene, contained no detectable polyP. *E. coli* CA38/pBC29, which carried the *ppk* gene only on a high-copy plasmid, contained 20-fold more polyP than did *E. coli* CA38/pBC9, which carried the *ppk* and *ppx* genes on a high-copy plasmid.

Cadmium tolerance studies. Studies were conducted to determine the effect of cadmium on cell growth. The strains were grown overnight (approximately 10 generations) to the mid-exponential phase in MOPS-glucose medium with no cadmium. The exponentially growing cells were diluted into medium with the same composition but containing various concentrations of cadmium chloride. Care was taken to dilute cells to the same optical density at 600 nm in each flask. The results of studies of the growth of these strains on cadmium concentrations up to 10 ppm are shown in Fig. 2. For strains CA38/pUC18 and CA38/pBC29, the growth rate decreased with increasing cadmium concentrations. In contrast, increasing cadmium concentrations up to 2 ppm showed no apparent effect on the growth of strain CA38/pBC9. A cadmium concentration of 4 ppm significantly slowed the growth of all three strains, more so for CA38/pBC29 than for CA38/pBC9 and CA38/pUC18, and a concentration of 10 ppm inhibited the growth of all strains. The doubling times in the exponential

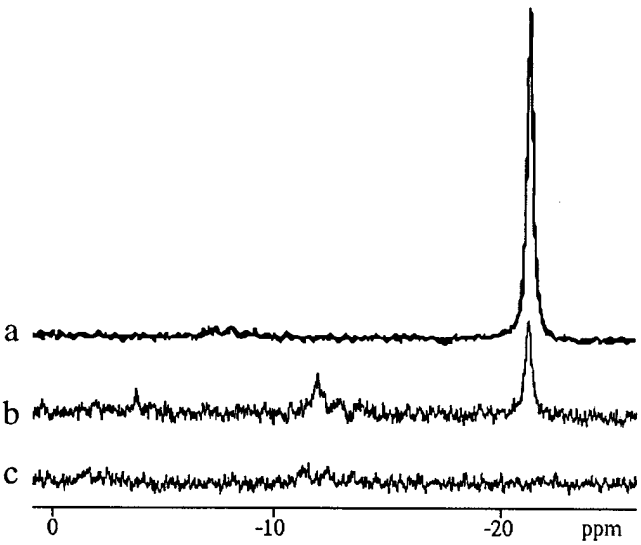

FIG. 1. Analysis of polyP extracts by ^{31}P nuclear magnetic resonance spectroscopy. The peak at −22 ppm corresponds to the internal residues of polyP. a, *E. coli* CA38/pBC29; b, *E. coli* CA38/pBC9; c, *E. coli* CA38/pUC18.

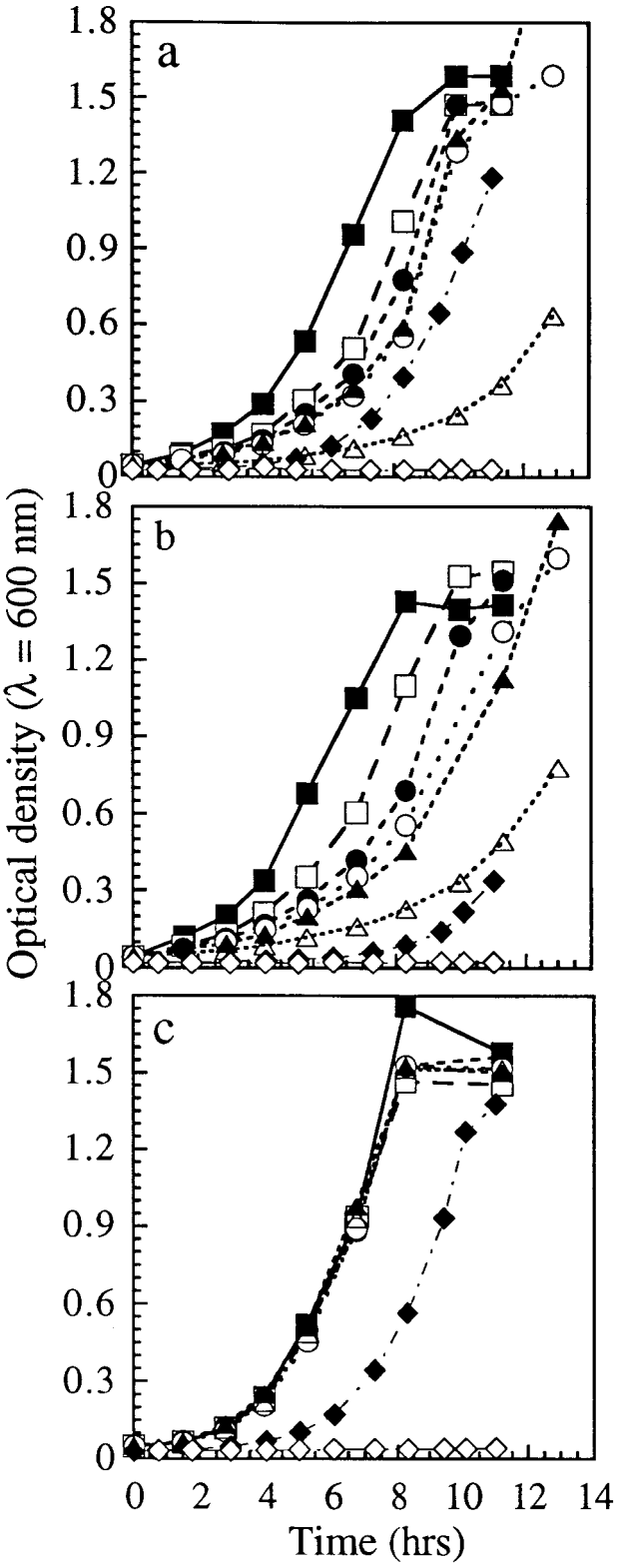

FIG. 2. Growth of genetically manipulated strains in medium containing cadmium. Time zero is the time of dilution into cadmium-containing medium. Growth was assessed by measuring optical density at 600 nm. Symbols: ■, Cd^{2+} at 0 ppm; □, Cd^{2+} at 0.25 ppm; ●, Cd^{2+} at 0.5 ppm; ○, Cd^{2+} at 0.75 ppm; ▲, Cd^{2+} at 1.0 ppm; △, Cd^{2+} at 2.0 ppm; ◆, Cd^{2+} at 4.0 ppm; ◇, Cd^{2+} at 10.0 ppm. a, *E. coli* CA38/pUC18; b, *E. coli* CA38/pBC29; c, *E. coli* CA38/pBC9.

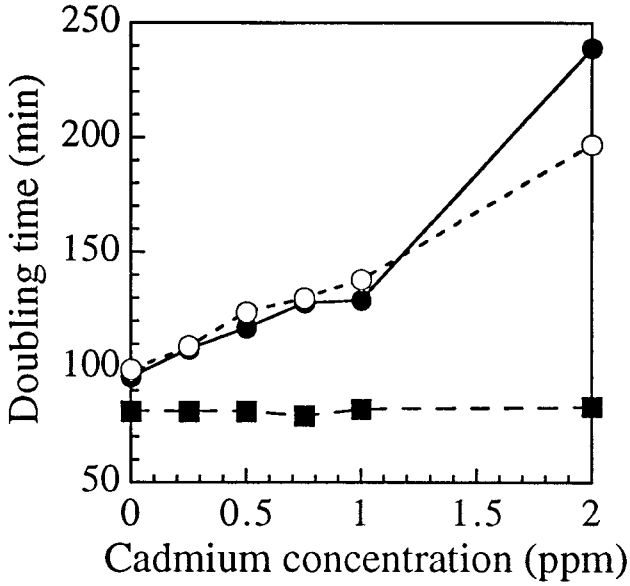

FIG. 3. Effect of cadmium on cell doubling time. The doubling times of cells during growth on cadmium (0 to 2 ppm) were determined from the data in Fig. 2. Symbols: ●, *E. coli* CA38/pUC18; ○, *E. coli* CA38/pBC29; ■, *E. coli* CA38/pBC9.

growth regions were determined from these graphs. We plotted the doubling times for cadmium concentrations of up to 2 ppm, the range in which the doubling times differed most significantly (Fig. 3).

The data indicate that polyP plays an indirect role in heavy metal tolerance. Rather than the level of polyP, as has been suggested previously, it may be the ability to hydrolyze polyP that is important for heavy metal tolerance in *E. coli. E. coli* CA38/pBC29, which carried only *ppk* on a high-copy plasmid and contained the highest level of polyP of any strain studied, did not show significantly better cadmium resistance than *E. coli* CA38/pUC18, which had the lowest polyP level of any strain studied. In contrast, *E. coli* CA38/pBC9, which was able to synthesize and degrade polyP, was significantly more resistant to cadmium than were the other two strains. These results are supported by the work of Hashemi and coworkers (9), who found that even though cells with large intracellular pools of polyP prior to metal exposure were more resistant to metal toxicity than cells with small polyP pools, the metals were not localized on the polyP bodies.

One possible mechanism for the increased cadmium tolerance of *E. coli* CA38/pBC9 is degradation of polyP by PPX and precipitation of cadmium inside the cell or transport of cadmium-phosphate out of the cell. It has been shown that PPX has maximum activity in the presence of a high salt concentration (170 mM) (4, 10). A high intracellular metal concentration may stimulate hydrolysis of polyP, which could then lead to intracellular precipitation of the metal or to efflux of a metal-phosphate complex from the cell. Indeed, the efflux of various cations from cells during the degradation of polyP has been reported (24), as has the degradation of polyP during heavy metal challenges (30).

Recently, Van Veen and coworkers found that the phosphate inorganic transport system in *E. coli* and *Acinetobacter johnsonii* 210A transports metal-phosphate complexes into or out of the cell, depending on the state of the cellular phosphate and energy metabolism (26). They suggested that such a system is used to take up divalent cations, such as magnesium, and

phosphate from the environment or to pump metal-phosphate complexes out of cells to generate a proton motive force (27). Such a system could be used by the cells to balance their internal metal content and to excrete toxic cations (25).

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