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Polyamines Affect Polyphosphate Accumulation in *Escherichia coli*

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In the current studies, we showed that a *speABC* and *cadA* mutant of *Escherichia coli,* which fails to produce polyamines such as putrescine, spermidine, and cadaverine, accumulates reduced levels of polyphosphate (polyP) under conditions of amino acid starvation. This was reversed by supplementation of the culture medium with polyamines. In addition, a *speG* mutant, which accumulates polyamines, increased levels of polyP, suggesting that polyamines affect polyP accumulation. Although polyP granules are often found in cellular inclusions containing calcium, those isolated from *E. coli* did not contain calcium. We found that these polyP granules soon disappear when they are placed in a buffer but that they are stabilized by the presence of polyamines. We concluded that polyamines interact with polyP and affect polyP accumulation.

Key words: Polyphosphate, polyamine, *Escherichia coli*, polyphosphate granule

1. Introduction

Increased input of inorganic phosphate (P_i) into lakes, bays, and other surface waters causes the nuisance growth of phytoplankton, called an algal bloom[6]. Hence, considerable attention has been paid to efficient removal of P_i from wastewater[6,28]. In traditional sewage works, 20% to 40% of P_i in wastewater is typically removed in the sludge by biological conversion to organic phosphate such as DNA, RNA, and phospholipid. Improvement of the ability to accumulate phosphorus compounds in the sludge should therefore help in the removal of P_i from wastewater.

Inorganic polyphosphate (polyP) is a linear polymer of many tens or hundreds of P_i molecules connected by high-energy bonds[14,15]. PolyP is found in a wide range of prokaryotes and eukaryotes[14,15]. PolyP has various biological functions, for example, as a P_i reservoir[14,15], an alternative source of high-energy bonds[14,15], as a buffer against alkaline conditions and metal accumulation[23], and as a regulator for transcription[14] and proteolysis[18]. PolyP has an osmotic advantage over free P_i for accumulation of phosphorus in cells. Therefore, enhanced biological phosphorus removal is based on the use of bacteria capable of accumulating polyP.

PolyP kinase (PPK), a tetramer of 80-kDa subunits, is responsible for the processive synthesis of long-chain polyP (750 P_i residues) in *Escherichia coli*[14]. PPK catalyzes the reversible reaction of the γ-terminal P_i of ATP to polyP[14]. So far, *phoU*, *spoT*, and *relA* genes have been identified as regulators of polyP accumulation[16,21]. A *phoU* mutant, which derepresses the phosphate regulon, accumulates polyP[21]. Constitutive expression of the PstSCAB P_i uptake system in the *phoU* mutants is responsible for the elevated levels of polyP[21]. On the other hand, the level of polyP in *E. coli* is very low during the exponential phase but increases up to 1000-fold in response to amino acid starvation[16]. An *E. coli relA spoT* mutant that fails to produce guanosine penta- and tetra-phosphate (pppGpp and ppGpp) is deficient in the accumulation of polyP in response to amino acid starvation[16]. It was found that the stringent factors pppGpp and ppGpp strongly inhibit polyphosphatase PPX[16]. PPK and PPX activities are constitutively expressed, but when pppGpp and ppGpp build up in the cells during amino acid starvation, polyP may accumulate due to inhibition of PPX.

Polyamines, polycationic compounds present in all living organisms, have been implicated in a wide variety of biological reactions, including nucleic acid and protein synthesis[3,10]. Polyamines are thought to participate in these cellular processes through binding to DNA and RNA[10,11]. Polyamine content is regulated by its biosynthesis, degradation, uptake, and excretion[9,22]. A polyamine-deficient strain of *E. coli* has been obtained as a result of deletions in *speA* (arginine decarboxylase), *speB* (agmatine ureohydrolase), *speC* (ornithine decarboxylase), and *cadA* (cadavarine biosynthesis)[13,29]. Although this strain lacks putrescine, spermidine, and cadaverine, it is still able to slowly but indefinitely grow on polyamine-deficient media[13,29]. In the presence of polyamines in the medium, an *speABC* and *cadA* mutant can take up polyamines and grow well. On the other hand, a spermidine acetyltransferase gene (*speG*) mutant that fails

to degrade polyamine accumulates approximately 3-fold higher levels of spermidine when grown in a medium containing spermidine[5]. We speculated that polyamines interact not only with DNA and RNA but also polyP, and we demonstrated that polyamines affect polyP accumulation under conditions of amino acid starvation.

2. Materials and Methods

2.1. Plasmid construction

A 2.6-kb *Kpn*I fragment containing *ppk* of pBC10 was inserted into the *Kpn*I site of pMW119. The orientation of the *ppk* insertion in the resulting plasmid pMWppk was opposite the lac promoter of pMW119[17].

2.2. Bacterial strains and growth conditions

E. coli speABC, *cadA*, and *speG* mutants were constructed according to the one-step gene inactivation method described by Datsenko and Wanner[4]. First, DNA fragments containing target and antibiotic genes were prepared by polymerase chain reaction using the primers listed in Table 1. These DNA fragments were then introduced into *E. coli* and replaced with the chromosomal genes. The *speABC cadA* mutant was grown in a 2xYT rich medium (1.6% peptone, 1.0% yeast extract, and 0.5% NaCl) with shaking at 37°C for 12–18 h. This culture was diluted in a MOPS-minimal medium (22.2 mM glucose, 40 mM potassium morpholinopropane sulfonate [pH 7.2], 50 mM NaCl, 9.52 mM NH_4Cl, 4 mM Tricine, 2 mM K_2HPO_4, 0.52 mM $MgCl_2$, 0.28 mM K_2SO_4, 0.01 mM $FeSO_4$, 0.0005 mM $CaCl_2$, and trace metals)[17] containing 2% casamino acid and 0.4 M sorbitol and then cultivated overnight. To deplete endogenous polyamines, this cultivation was repeated, and the culture was inoculated into the MOPS-minimal medium containing 2% casamino acid and 0.4M sorbitol. The fresh medium was supplemented with 1 mM putrescine and spermidine as necessary. After a 6-h incubation, the *E. coli speABC cadA* mutant was collected by centrifugation. The cell pellet was washed once with the MOPS-minimal medium containing 0.4 M sorbitol and resuspended in the same medium (amino acid-starved condition). The *E. coli speG* mutant was grown to mid-log phase in the 2xYT medium with shaking at 37°C and shifted to the MOPS-minimal medium. PolyP granules were observed under fluorescent microscopy (BX-50, Olympus) after staining with 4',6-diamidino-2-phenylindole (DAPI). PolyP was recovered with silicate glass from cells lysed with guanidine isothiocyanate and determined by a two-enzyme assay[1].

2.3. Preparation of polyP granules from *E. coli*

The *E. coli phoU* mutant was grown to mid-log phase in the 2xYT medium with shaking at 37°C and shifted to the MOPS-minimal medium for 2 h to cause the accumulation of a large amount of polyP-containing granules[21]. PolyP granules were prepared essentially as described by Jacobson[12]. Freeze-dried cells (0.1 g), zirconia beads (diameter 2 mm; 3.5 g), and carbon tetrachloride (400 µl) were mixed in a 2-ml tube. The suspension was homogenized twice for 30 s using a Micro Smash (Tomy, Tokyo, Japan) at a speed of 4000 rpm in a cold room. PolyP granules were precipitated by centrifugation at 13,000×*g* for 10 min. The cell debris was not precipitated under this condition. The pellet containing polyP granules was washed several times with carbon tetrachloride and dried. PolyP granules were observed by scanning electron microscopy, and their chemical composition was analyzed by electron microscopy coupled with energy-dispersive X-ray spectroscopy (EDAX; Nihon Denshi).

2.4. *In vitro* preparation of polyP granules

PolyP granules were formed by mixing 50 mM polyP (average chain-length=65; Sigma), 57 mM KCl, and 39 mM $MgCl_2$. PolyP granules were collected by centrifugation at 13,000×*g* for 5 min and washed five times with a solution containing 57 mM KCl and 39 mM $MgCl_2$.

Table 1. Primers used for gene disruption.

Gene	DNA sequence
speAB	ACTGTTTTACACTTAATAAAATAATTTGAGGTTCGCTATGATTCC-GGGGATCCGTCGACC (forward)
	TGCGCATCGCATCTGGTGCTTACTCGCCCTTTTTCGCCGCTGTAG-GCTGGAGCTGCTTCG (reverse)
speC	GGCGGTCGGAGCTGGTGACCAGTTTGACCCATATCTCATGATTCC-GGGGATCCGTCGACC (forward)
	TGACCCGTTTTTTTTATTCTTACTTCAACACATAACCGTATGTAG-GCTGGAGCTGCTTCG (reverse)
cadA	GTGTTGGGAGGGGCCTTTTTTACCTGGAGATATGACTATGATTCC-GGGGATCCGTCGACC (forward)
	CTTCCCTTGTACGAGCTAATTATTTTTTGCTTTCTTCTTTTGTAG-GCTGGAGCTGCTTCG (reverse)
speG	TAACCTGTTATTGATTTAAGGAATGTAAGGACACGTTATGATTCC-GGGGATCCGTCGACC (forward)
	CGATCGATTATTATTAATGCTATTGTGCGGTCGGCTTCAGTGTAG-GCTGGAGCTGCTTCG (reverse)

2.5. Stability of polyP granules

PolyP granules prepared *in vitro* were dispersed in 100 mM sodium hydroxyetylpiperazine ethanesulphonate (HEPES-NaOH, pH 7.2) containing each polyamine (putrescine, spermidine, cadaverine), or ammonium chloride at room temperature. After the indicated time, polyP granules were precipitated at 13,000×*g* for 3 min. PolyP was determined as P_i by the ascorbic acid method after hydrolysis in 1 N HCl at 100°C for 7 min[2,7].

3. Results and Discussion

3.1. Polyamines affect polyP accumulation in *E. coli*

PolyP accumulated in response to amino acid starvation in *E. coli* (Fig. 1A). PolyP is a P_i polymer with some similarity to RNA and DNA, and, therefore, it is reasonable that polyP readily interacts with polycationic compounds including polyamines. To determine whether polyamines affect polyP accumulation under conditions of amino acid starvation, we constructed a *speABC* and *cadA* mutant that fails to produce polyamines[13,29]. The intracellular levels of polyamines in this mutant can be restored by the addition of polyamines to the medium. The levels of polyP in this mutant were also partially restored by addition of polyamines to the medium under conditions of amino acid starvation (Fig. 1A). In addition, introduction of a low-copy plasmid encoding *ppk* (pMWppk) increased the levels of polyP. Under conditions of amino acid starvation, an *speABC* and *cadA* mutant carrying pMWppk accumulated more polyP in the presence than in the absence of polyamines in the medium (Fig. 1B). On the other hand, accumulation of polyamines by a *speG* mutant was enhanced three-fold by the addition of polyamines to the medium.

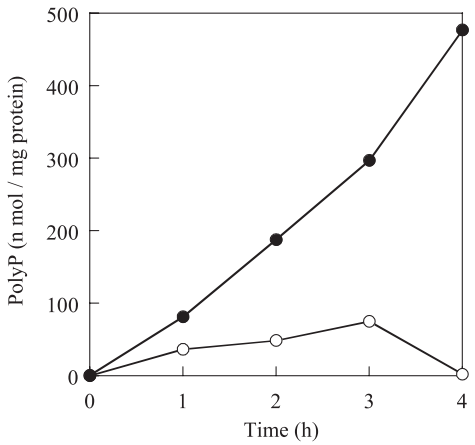

Fig. 2. PolyP accumulation in a *speG* mutant and the wild type under conditions of amino acid starvation in the presence of polyamines.
Intracellular polyP concentrations were measured for *E. coli* MG1655 (open circles) and a *speG* mutant (closed circles) at the indicated times after shifting to MOPS medium containing polyamines (1 mM putrescine and 1 mM spermidine).

This mutant also accumulated more polyP than the wild type (Fig. 2). After 3 h, the wild type restarted to grow under this condition and its polyP accumulation reduced (Fig. 2). However, this mutant could not adapt to amino acid starvation and thus polyP accumulation might prolong even after 3 h. These results suggested that polyamines affect polyP accumulation.

3.2. Analysis of polyP granules prepared from *E. coli*

PolyP is often found in cellular inclusions known as polyP granules or volutin granules[19,25,26,27]. Here, we isolated polyP granules from an *E. coli phoU* mutant that accumulates 1000-fold higher levels of polyP than the wild type.

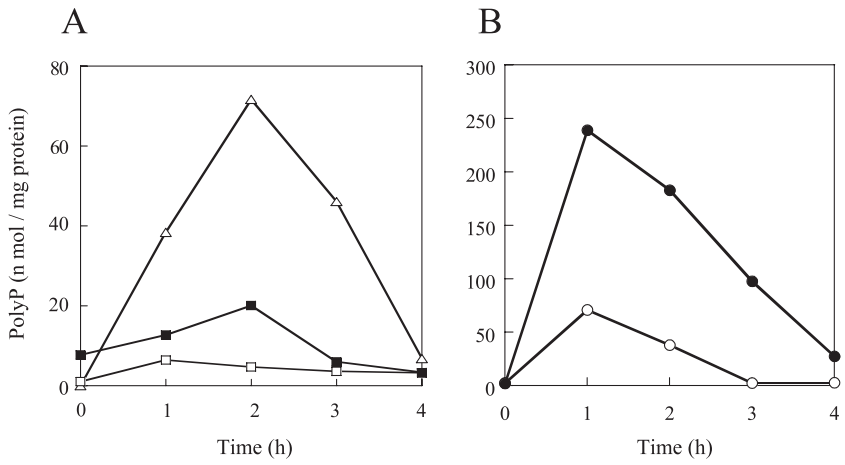

Fig. 1. PolyP accumulation in a *speABC cadA* mutant under conditions of amino acid starvation and in the presence and absence of polyamines.
(A) Intracellular polyP concentrations were measured in the *E. coli* wild type at the indicated times after shifting to amino acid-starved conditions (open triangles). The polyP concentrations were also measured in an *E. coli speABC cadA* mutant at the indicated times after shifting to amino acid-starved conditions with (closed squares) or without (open squares) added polyamines (1 mM putrescine and 1 mM spermidine). (B) The same experiment was performed in the *E. coli speABC cadA* mutant carrying pMWppk in the presence (closed circular) or absence (open circular) of polyamines.

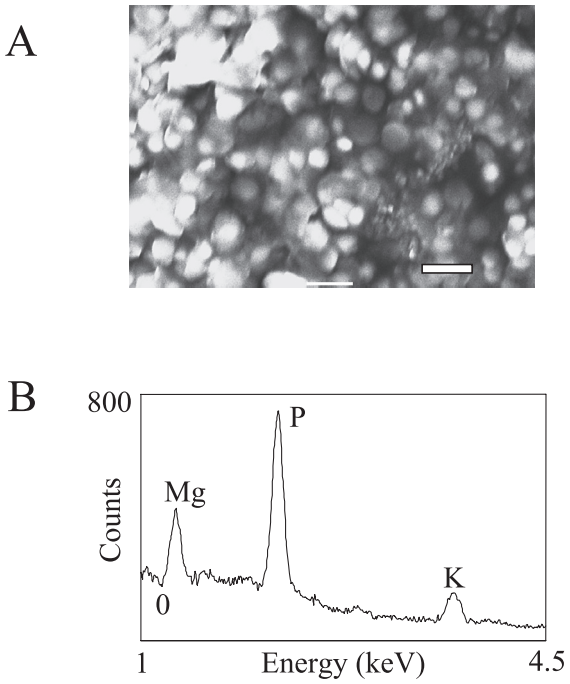

Fig 3. Scanning electron microscopy (A) and EDAX spectra (B) of polyP granules prepared from *E. coli* cells.
(A) PolyP granules extracted from *E. coli* with carbon tetrachloride were evaporated on the stage, coated with carbon, and then observed by scanning electron microscopy. Bar=2 μm. (B) The elemental composition of polyP granules was analyzed by EDAX.

We examined the polyP granules by microscopy and by scanning electron microscopy with EDAX (Fig. 3). EDAX analysis revealed that polyP granules contained phosphorus, magnesium, and potassium in a ratio of 1.0:0.31:0.19. Although polyP granules are often found in cellular inclusions containing calcium[14], those isolated from *E. coli* did not contain calcium.

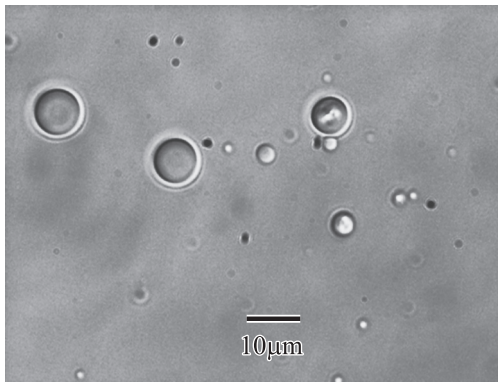

Fig. 4. Microscopic analysis of synthesized polyP granules.
The synthesized polyP granules were observed under phase-contrast microscopy.

We prepared polyP granules *in vitro* by mixing polyP, KCl, and $MgCl_2$ as to approximately the same elemental ratio as the polyP granules prepared from *E. coli* cells. The synthetic polyP granules were spherical with diameters from several hundreds of nanometers to tens of micrometers (Fig. 4). However, these synthetic polyP granules dissolved within 50 min when they were placed in HEPES buffer (data shown in Fig. 5B).

3.3. Polyamines protect polyP granules from dissolution

We suspected that polyamines protect polyP granules from dissolution in *E. coli*. We found that the polyamines such as putrescine, spermidine, and cadaverine protect the polyP granules from dissolution but that ammonium chloride does not (Fig. 5A). Igarashi and Kashiwagi predicted that 12.5 mM putrescine and 0.26 mM spermidine exist in a free form in *E. coli*[20]. The polyP granules were completely protected against dissolution by a combination of 12.5 mM putrescine and 0.26 mM spermidine (Fig. 5B).

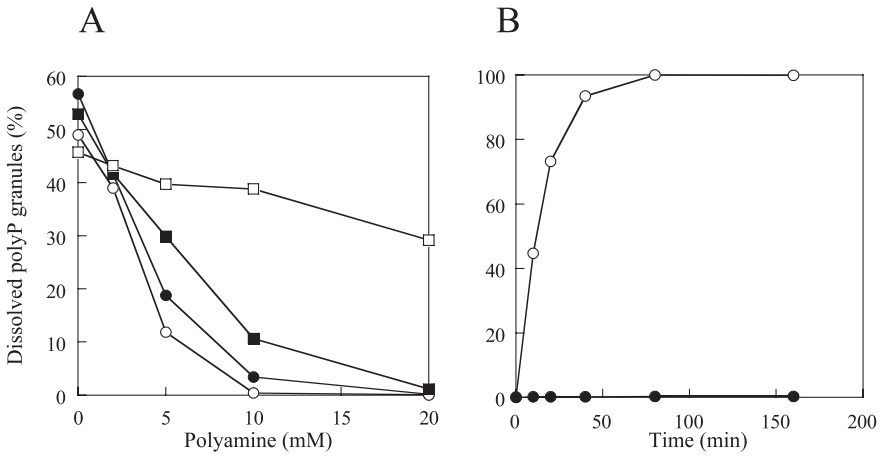

Fig. 5. Polyamines stabilize polyP granules.
(A) PolyP granules prepared *in vitro* (0.5 mg) were precipitated by centrifugation and placed in HEPES buffer containing various concentrations of putrescine (closed circular), spermidine (open circular), cadaverine (closed square), or ammonium chloride (open square). Dissolved polyP could no longer be precipitated by centrifugation. The percentage of dissolved polyP after 10-min incubation vs. total polyP granules was plotted as a function of the polyamine concentration. (B) PolyP granules prepared *in vitro* (0.5 mg) were incubated in HEPES buffer in the presence (closed) and absence (open) of 12.5 mM putrescine and 0.26 mM spermidine. The percentage of dissolved vs. total polyP granules was plotted as a function of the incubation time.

We prepared polyP granules *in vitro* by mixing polyP, KCl, and $MgCl_2$ in the presence of 10 mM spermidine. The synthetic polyP granules were very stable even when they were placed in HEPES buffer in the absence of polyamine (data not shown). These results suggested that the polyamines also interact with polyP when polyP granules are formed.

PolyP was first identified in microorganisms as metachromatic granules that are stained pink with basic blue dyes, and they were originally referred to as "volutin granule"[14]. PolyP granules from unicellular eukaryotes (*e.g.*, *Chlamydomonas reinhardtii*[25] and *Dictyostelium discoideum*[19]), a number of human pathogens (*e.g.*, malaria parasites and trypanosomatids[26]), and human platelets[27] were recently found to be surrounded by a membrane with a number of pumps and exchangers and were named acidocalcisomes. Although polyP granules are often found in cellular inclusions containing calcium[14], those isolated from *E. coli* did not contain calcium. We found that these polyP granules soon disappeared when added to buffer, although they are stabilized by polyamines (putrescine, spermidine, and spermine), which are known to be present at millimolar concentrations in both prokaryotic and eukaryotic cells[3,10]. We concluded that polyamines may interact with polyP *in vivo* and therefore affect polyP accumulation.

Our findings also suggested that, conversely, polyP accumulation may affect the intracellular levels of free polyamine. A decrease or increase in polyamine content greatly diminishes cell growth in *E. coli*[8,24], and their intracellular levels are closely regulated by control of synthesis, degradation, uptake, and excretion[9,22]. In *E. coli*, approximately 50% of putrescine and 90% of spermidine exist as polyamine-RNA complexes, and they act at the level of translation to enhance the synthesis of OppA, Cya, RpoS, FecI, and Fis[11]. Further investigations are needed to clarify the role of polyP in polyamine metabolism.

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