APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2002, p. 4107–4110 Vol. 68, No. 8
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.8.4107–4110.2002

Accumulation of Inorganic Polyphosphate in *phoU* Mutants of *Escherichia coli* and *Synechocystis* sp. Strain PCC6803

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Received 14 February 2002/Accepted 7 May 2002

The biological process for phosphate (P_i) removal is based on the use of bacteria capable of accumulating inorganic polyphosphate (polyP). We obtained *Escherichia coli* mutants which accumulate a large amount of polyP. The polyP accumulation in these mutants was ascribed to a mutation of the *phoU* gene that encodes a negative regulator of the P_i regulon. Insertional inactivation of the *phoU* gene also elevated the intracellular level of polyP in *Synechocystis* sp. strain PCC6803. The mutant could remove fourfold more P_i from the medium than the wild-type strain removed.

Inorganic phosphate (P_i) is recognized as one of the major nutrients causing eutrophication of lakes, bays, and waterways (18). Considerable attention has been paid to effective P_i removal from wastewater (6). Many bacteria are capable of accumulating excess P_i in the form of inorganic polyphosphate (polyP), which is a linear polymer of hundreds of P_i residues linked by high-energy phosphoanhydride bonds (7, 9, 10). Improvement of the ability to accumulate polyP contributes to increased P_i removal from wastewater (18).

Previously, we have demonstrated genetic improvement of polyP accumulation in *Escherichia coli* (8). High levels of accumulated polyP were achieved by increasing the dosage of the *E. coli* genes encoding polyP kinase (*ppk*) and the P_i-specific transport system (*pstSCAB*). The *E. coli* recombinant accumulated approximately 16% of its dry weight as phosphorus (P) (49% as P_i) (8). Over 60% of cellular P was stored in the form of polyP in the genetically engineered *E. coli* strain. However, growth of the *E. coli* recombinant was severely limited in minimal medium (8). In addition, this recombinant released polyP back into the medium when it accumulated high levels of polyP. In this paper, we report that a mutation in the *phoU* gene, which encodes a negative regulator of the P_i regulon (3, 21), led to high levels of accumulated polyP in *E. coli*. *phoU* mutants could be easily screened on agar plates containing 5-bromo-4-chloro-3-indolylphosphate (XP) after *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis. Therefore, isolating *phoU* mutants seems to be a useful way to improve the ability of bacteria to accumulate polyP. To show whether this method is effective in another bacterium, we performed insertional inactivation of the *phoU* gene in *Synechocystis* sp. strain PCC6803 and showed that the intracellular level of polyP increased in the mutant.

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Isolation of *E. coli* mutants. The levels of polyP in *E. coli* MG1655 were very low (less than 1 nmol of P_i residues/mg of protein) when the organism was grown on a rich medium (11). PolyP was recovered with silicate glass from cells lysed with guanidine isothiocyanate, and the polyP content was determined by a two-enzyme assay (4). PolyP was first converted to ATP by polyphosphate kinase, and then the amount of ATP was measured by a bioluminescence assay. We first selected alkaline phosphatase constitutive mutants, which could form blue colonies on agar plates containing XP (50 mg/liter) under P_i-sufficient conditions after *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis (17). One of 150 mutants, designated MT4, accumulated a large amount of polyP (approximately 100 nmol of P_i residues/mg of protein). The levels of polyP were at least 100-fold higher than the levels in the wild-type strain, MG1655.

To assess P_i transport (14), the *E. coli* mutants were grown overnight in MOPS (morpholinepropanesulfonic acid) medium (12) containing either 0.1 mM P_i (P_i limiting) or 2 mM P_i (P_i sufficient). Cells were harvested by centrifugation at 8,000 $\times g$ for 5 min and washed with HEPES buffer (13). Assays were started by adding $^{32}P_i$ to a final concentration of 2.5 μM, and the amount of $^{32}P_i$ incorporated into the cells was determined by using a scintillation counter (Packard). Only MT4 exhibited P_i-specific transport even when the organisms were grown with excess P_i (Fig. 1).

Analysis of the mutation of MT4. An *E. coli* DNA library based on the SuperCos plasmid (Stratagene) was introduced into MT4 by transformation (17). Approximately 200 transformants were examined for the ability to accumulate polyP in 2×YT medium (17). One transformant reduced the levels of polyP to the level in the wild-type strain. This transformant carried a recombinant plasmid containing a 30-kb DNA fragment of the *E. coli* chromosome. Nucleotide sequence analysis of the 30-kb insert revealed that this fragment contained the *pst* and *bgl* operons which were located at 84 min on the *E. coli* linkage map (5). Subcloning and complementation analysis revealed that a 3.0-kb *Eco*RI fragment, carrying the entire *phoU* gene, could complement the mutation of MT4. The chromosomal *phoU* gene of MT4 was amplified by PCR with prim-

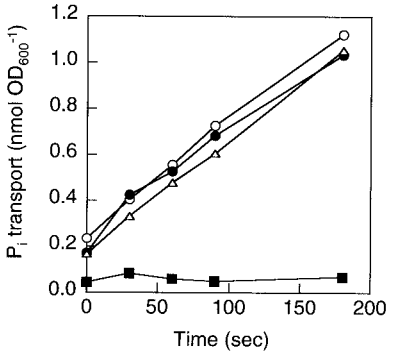

FIG. 1. P_i transport as assessed by using P_i-sufficient MG1655 (■), P_i-limited MG1655 (●), P_i-sufficient MT4 (△), and P_i-limited MT4 (○) cultures. Cells were grown in MOPS medium containing either 2 mM P_i (P_i sufficient) or 0.1 mM P_i (P_i limited). OD, optical density at 600 nm.

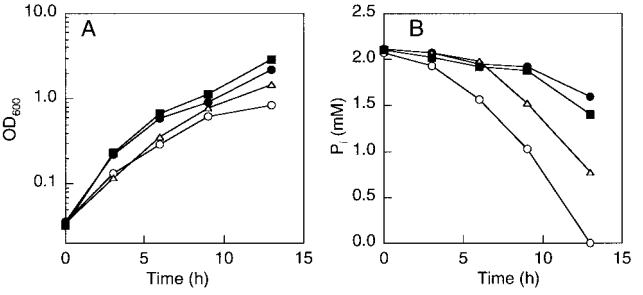

FIG. 2. Growth (A) and P_i uptake (B) of *E. coli* MG1655, MG1655(pBC29), MT4, and MT4(pBC29). Strains MG1655 (■), MG1655(pBC29) (●), MT4 (△), and MT4(pBC29) (○) were grown in MOPS medium containing 2 mM P_i at 28°C. OD_{600}, optical density at 600 nm.

ers EU1 (5′-ATTGGGATTTGTCTGGTGAA-3′) and EU2 (5′-AGAAGACTACATCACCGGTC-3′) and cloned into the pGEM-T vector (Promega). Nucleotide sequence analysis showed that the 29th codon of the *phoU* gene was changed from a glycine codon to an aspartic acid codon in MT4. We again selected a polyP-accumulating mutant from the mutants producing alkaline phosphatase constitutively. In this mutant, the 83rd codon of the *phoU* gene was changed from an alanine codon to a threonine codon. These results indicated that mutation of the *phoU* gene resulted in elevated intracellular levels of polyP in *E. coli*.

To further confirm that *phoU* mutants could accumulate high levels of polyP, the chromosomal *phoU* gene of MG1655 was disrupted by inserting a kanamycin resistance (Km^r) gene cassette into the wild-type gene. A 0.8-kb DNA fragment containing the entire *phoU* gene was amplified by PCR with the EU1 and EU2 primers and inserted into the pGEM-T vector. The resultant plasmid was digested with *Cla*I and ligated with the Km^r gene cassette of pUC4K (Amersham-Pharmacia), and an *Eco*RI fragment containing a disrupted *phoU* gene was inserted into an *Eco*RI site of pGP704 containing the *sacB* gene (16). This plasmid was introduced into *E. coli* S17-1 and then transferred into MG1655 by transconjugation (22). Transconjugants were selected on agar plates containing 5% sucrose and kanamycin (50 mg/liter). Disruption of the chromosomal *phoU* gene was confirmed by Southern hybridization. As expected, the insertional *phoU* mutant accumulated high levels of polyP (approximately 400 nmol of P_i residues/mg of protein).

One might assume that *ppk* is a member of the P_i regulon in *E. coli* and that derepressed expression of this gene in the *phoU* mutants results in polyP accumulation. However, no significant increase in the level of polyphosphate kinase was observed under P_i-limited conditions in *E. coli* (Morohoshi and Kuroda, unpublished data). Furthermore, expression of a single-copy *ppk-lacZ* transcriptional fusion did not increase under P_i limitation conditions (L. Zhou and B. L. Wanner, personal communication). P_i transport is rate limiting for polyP accumulation in *E. coli* (8). A *phoU* mutant exhibited P_i-specific transport even under P_i-excess conditions (Fig. 1). We transferred a Δ(*pstSCAB-phoU*)::*km* mutation from BW17335 (20) to the wild-type strain, MG1655, by using P1 phage. This mutant failed to accumulate polyP (0.5 nmol/mg of protein). Consequently, it is likely that constitutive expression of P_i-specific transport (PstSCAB) is responsible for the elevated levels of polyP in the *phoU* mutants.

Although MT4 grew relatively slowly, it removed twofold more P_i from the medium than the wild type removed (Fig. 2). Introduction of multicopy plasmid pBC29, which contains the *E. coli ppk* gene (1), into the wild-type strain, MG1655, did not increase P_i removal significantly (Fig. 2). By contrast, MT4(pBC29) removed about twofold more P_i than MT4 removed (Fig. 2). The P content of MT4(pBC29) reached approximately 9% on a dry weight basis (27% as P_i) and was about sixfold greater than that of the wild-type strain, MG1655. PolyP granules were detected in MT4(pBC29) when the cells were observed by fluorescent microscopy after they were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (2) (Fig. 3).

***phoU* mutant of *Synechocystis* sp. strain PCC6803.** A chromosomal *phoU* mutant of *Synechocystis* sp. strain PCC6803 was

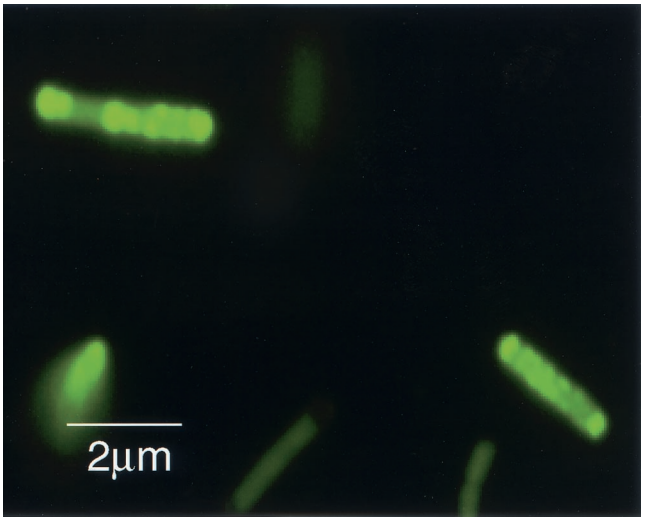

FIG. 3. PolyP granules in MT4(pBC29) detected by DAPI fluorescence. Strain MT4(pBC29) was grown overnight on 2×YT medium, stained with DAPI at a final concentration of 50 μg/ml, and observed with a fluorescence microscope (Olympus BX-40).

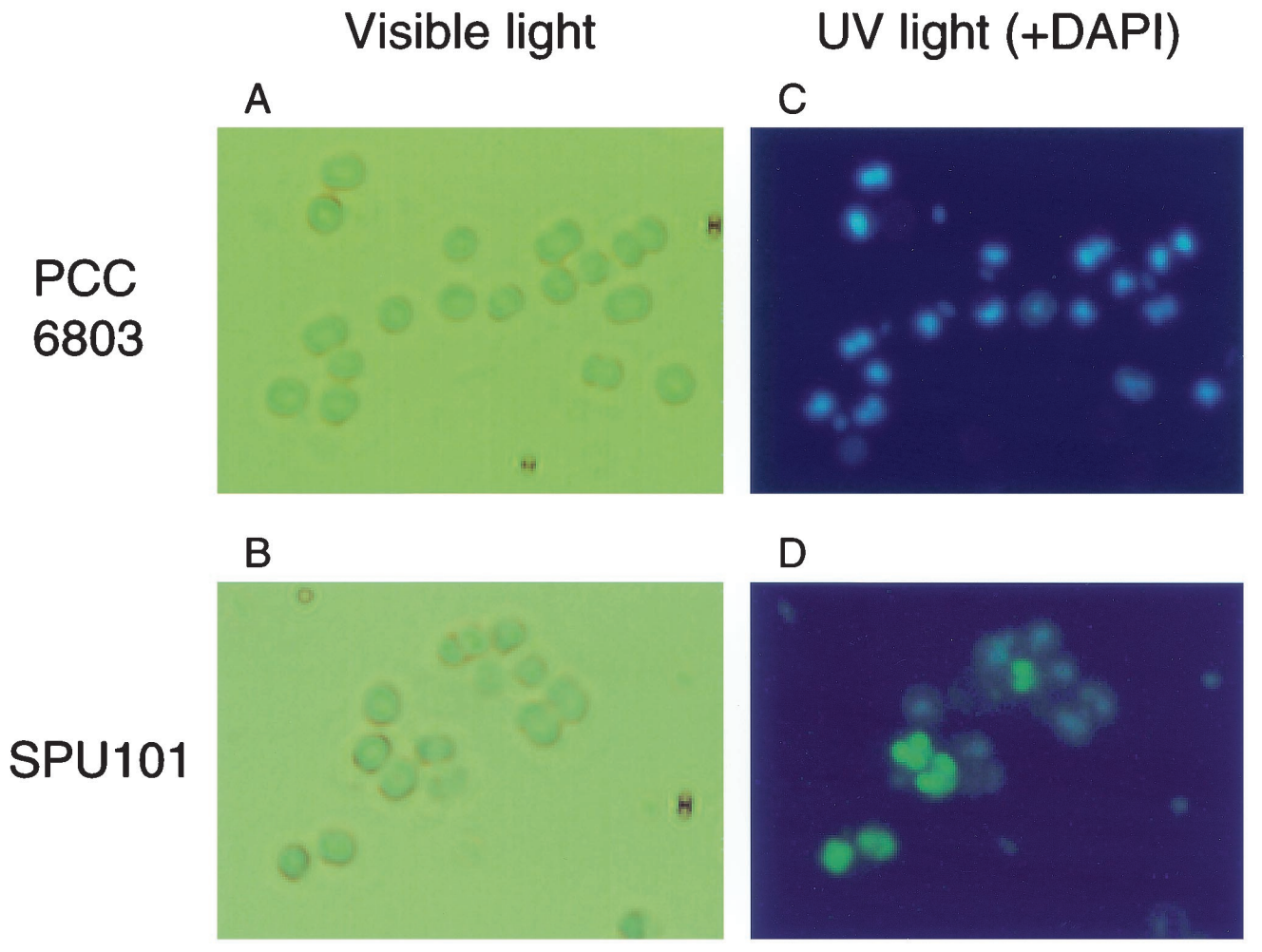

FIG. 4. PolyP granules in *Synechocystis* sp. strain PCC6803 and *phoU* mutant SPU101 detected by DAPI fluorescence. Strains PCC6803 and SPU101 were grown in BG-11 medium at 30°C under light (5,000 lx) with 1% CO_2 for 3 days and stained with DAPI. Cells were observed with either visible light (A and B) or UV light (C and D).

also constructed by inserting a Km^r gene cassette into the wild-type gene. A 2.0-kb DNA fragment, which carried the entire *phoU* gene, was amplified by PCR with primers SU1 (5′-GGTACCATCAACCTGATCGCCTAT-3′) and SU2 (5′-GCTACTGCTCCAGTCGACCCGAGT-3′) and cloned into pUC119. The resultant plasmid was digested with *Bgl*II, ligated to the Km^r gene cassette of pUC4K, and introduced into *Synechocystis* sp. strain PCC6803 by electroporation (17). Disruption of the chromosomal *phoU* gene was confirmed by Southern hybridization (data not shown). The *Synechocystis phoU* mutant, designated SPU101, accumulated about 15% as much polyP (on a dry weight basis) as P_i. As in *E. coli* MT4(pBC29), polyP particles were detected in SPU101 by DAPI fluorescence (Fig. 4). The total P content of the mutant strain reached a maximum of 6% on a dry weight basis. P_i uptake experiments were performed with growing cultures of PCC6803 and SPU101 (Fig. 5). Cultures were grown in BG-11 medium (19) with 1% CO_2 at 30°C under light (5,000 lx). Strain SPU101 removed about fourfold more P_i from the medium than the parental strain removed. The growth of SPU101 was almost equivalent to that of PCC6803.

We showed that a mutation of the *phoU* gene, which encodes a negative regulator of the P_i regulon, led to high levels of accumulated polyP in both *E. coli* and *Synechocystis* sp. Rao et al. previously reported that a *phoU* mutation had no effect

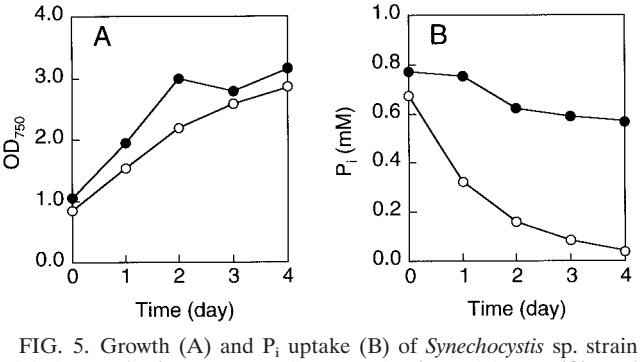

FIG. 5. Growth (A) and P_i uptake (B) of *Synechocystis* sp. strain PCC6803 and *phoU* mutant SPU101. Strains PCC6803 (●) and SPU101 (○) were grown in BG-11 medium at 30°C under light (5,000 lx) with 1% CO_2. OD_{750}, optical density at 750 nm.

on polyP accumulation (15). The *phoU* mutant of these authors probably had a secondary mutation in the Pst system, as described previously (20).

In enhanced biological P removal, sludge microorganisms accumulate 3 to 5% of their dry weight as P (18). Similar levels of accumulated polyP were observed with the *phoU* mutant of *Synechocystis* sp. strain PCC6803. In general, the levels of carbon sources in wastewater are relatively low. This is likely to limit P_i removal from wastewater by sludge microorganisms (18). Use of the *Synechocystis* mutant, which is able to accumulate polyP if light is present, may improve biological removal of P_i from wastewater.

We thank B. L. Wanner for a gift of the *phoU* and *pstSCAB* mutant and M. Ikeuchi for a gift of strain PCC6803.

This work was supported in part by the New Energy and Industrial Technology Development Organization (NEDO) and by the Research Institute of Innovative Technology for the Earth (RITE).

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