Role of Polyphosphate in Thermophilic Synechococcus sp. from Microbial Mats


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Synechococcus OS-B', a thermophilic unicellular cyanobacterium, recently isolated from the microbial mats in Octopus Spring (Yellowstone National Park), induces a suite of genes, including phosphatases and transporters, in response to phosphorus (P) starvation. Here we describe two different approaches to examine the ability of Synechococcus OS-B' to synthesize and break down polyphosphate (poly P), a key storage compound in many prokaryotes. First, we developed a transformation protocol to create mutants in the polyphosphate kinase (ppk), the major enzyme responsible for the synthesis of poly P. The ppk mutant exhibited a pleiotropic phenotype with defects in poly P accumulation, aberrant levels of Pho regulon transcripts, growth defects, and changes in cell size and exopolysaccharide levels, among others. Second, we measured transcripts of ppk and ppx (encoding the polyphosphatase) directly from mat samples and found that the levels varied dramatically over a diel cycle. We also used Western blot analysis to quantify levels of PPK and PPX and found that these enzymes differentially accumulated during the diel cycle. Levels of polyphosphate kinase peaked at night, while polyphosphatase levels were highest during the early morning. We hypothesize that the opposing activities of these two enzymes allow cells to store and utilize poly P to optimize growth over a diel cycle.

In the alkaline hot springs of Yellowstone National Park, photosynthetic microbes such as the thermophilic unicellular cyanobacterium Synechococcus sp. and filamentous anoxygenic phototrophs (FAPs) such as Chloroflexus sp. and Roseiflexus sp. (belonging to the phylum Chloroflexi) are found in the top green layer of the microbial mats, where they are spatially distributed along thermal, light, and oxygen gradients (1–3). The growth and survival of these populations are impacted by limiting nutrients, high temperatures, light levels, and phage predators (4, 5). The levels of most nutrients measured in the running water that flows above the mats are very low, so growth of the cells in these mats is likely to be primarily sustained by acquisition of recycled nutrients (4, 6). In such resource-poor environments, the ability to scavenge and store macronutrients such as N and P may be important for survival and carefully regulated by the cell. Most bacteria, including cyanobacteria, have several acclimation strategies to deal with P limitation as well as the ability to store P when it is available in excess (7, 8).

Two closely related isolates from these microbial mats, Synechococcus strain OS-A (GenBank accession number CP000239) and Synechococcus strain OS-B’ (GenBank accession number CP000240), have fully sequenced genomes and were used to explore the physiological responses of these thermophilic cyanobacteria to various growth conditions (9, 10, 11). To establish the ability of these organisms to grow under nutrient-depleted conditions, we initially measured the transcript levels of several members of the Pho regulon in the Synechococcus OS-B’ isolate when it was subjected to phosphorus deprivation (12). Many of these transcripts were strongly upregulated, which included periplasmic phosphatases such as PhoX (CYB_1988) and phosphate transporters (CYB_1074-CYB_1077 and CYB_1912-CYB_1915). Because the Synechococcus OS-B’ genome contained an entire operon encoding the C-P lyase and transporters (11), we also tested the ability of Synechococcus OS-B’ cells to grow using phosphonates, such as methyl or ethyl phosphonate, as a sole P source. Under these conditions, the genes encoding the C-P lyase and specific transport systems were induced (12). The closely related isolate, Synechococcus OS-A, which lacks the C-P lyase operon, induces specific phosphonatases to utilize phosphonates for growth. Furthermore, Synechococcus OS-B’ can utilize the organic carbon backbones of phosphonates for heterotrophic growth in the dark, whereas Synechococcus OS-A is unable to do so (13). Based on these results, it appears that the cyanobacteria of the hot springs have alternative and regulated metabolic strategies for cleaving and assimilating P from various sources in the resource-poor hot spring environment that allows for efficient biogeochemical cycling of both P and C.

In the environment, microorganisms face fluctuating and/or limiting levels of nutrients and have developed several strategies to deal with this situation (14, 15, 16). P is often a limiting nutrient in both terrestrial and marine environments (17), and organisms can detect external P levels via a two-component system (PhoB/PhoR). The PhoB/PhoR system regulates members of the Pho regulon, which allows the organism to efficiently scavenge P from the environment using a host of phosphatases, transporters, and other mechanisms (18, 19). On the other hand, if P is available in excess, microorganisms can store P in the form of polyphosphate (poly P), which is comprised of linear chains of phosphate residues linked by phosphoanhydride bonds (20). Poly P can appear as densely staining granules in cells and has been identified by microscopy in several bacterial species, including cyanobacteria.
Poly P is synthesized in bacteria from ATP by polyphosphate kinase 1 (PPK1, encoded by ppk) and degraded by exopolyphosphatases (PPX, encoded by ppx). Apart from simply being a storage form of P, poly P is also important for regulating a number of cellular processes such as stationary-phase survival and tolerance to osmotic, oxidative, and heat stress (20, 23). Currently, little is known about the role and importance of poly P in the extreme and nutrient-limited environments of the alkaline hot springs of Yellowstone National Park, and there are no reports describing poly P accumulation in thermophilic cyanobacteria. To examine the role and importance of poly P in the thermophilic cyanobacteria from the mat (9, 24), we have developed a genetic transformation strategy for *Synechococcus OS-B’*, which is described here. Stable transformants of *Synechococcus OS-B’* in which the *ppk* gene was insertionally inactivated with a thermostable kanamycin resistance cassette were obtained. The *ppk* mutant cells contained greatly reduced levels of poly P and exhibited pleiotropic phenotypes related to growth and physiology, suggesting that poly P plays an important role in thermophilic cyanobacteria. In a second in situ-based approach, we directly measured levels of *ppk* and *ppx* transcripts and protein levels of PPX and PPK in mat samples isolated over a diel cycle. These results indicated that levels of PPK and PPX transcripts and polypeptides vary significantly over a 24-h period, as we have demonstrated for other pathways such as those involved in photosynthesis, respiration, nitrogen fixation, fermentation, and oxidative stress (25–27). The implications of these diurnal changes are discussed in the context of optimizing growth under fluctuating environmental conditions.

**MATERIALS AND METHODS**

**Reagents.** [γ-32P]ATP (Amersham Biosciences, Piscataway, NJ), protein size markers (Bio-Rad, Hercules, CA), restriction enzymes (New England BioLabs, Ipswich, MA), poly P (types poly P₃₀ and poly P₇₀), apryrase, and common chemicals (Sigma-Aldrich, St. Louis, MO) were used. Poly P containing 35 phosphate residues (poly P₃₅), poly P₇₀, poly P₉₀, and poly P₇₅₀ along with polyphosphatase isolated from *Saccharomyces cerevisiae*, and polyclonal rabbit anti-Ec-PPK1 and anti-Pa-PPX antibodies were gifts from the A. Kornberg laboratory, Stanford University.

**Culture conditions.** Cultures of *Synechococcus OS-B’* (designated CIW 10) were grown at 50°C in liquid medium D (28) supplemented with CIW 10) were grown at 50°C in liquid medium D (28) supplemented with CIW 10) were grown at 50°C in liquid medium D (28) supplemented with ****

**Extraction and quantification of poly P.** Acid-soluble and salt-soluble poly P fractions were extracted at 4°C with 0.5 N HClO₄ and a saturated solution of NaClO₄ in 1 N HClO₄, respectively. The remaining bio mass was treated with 0.5 N HClO₄ for 30 min at 90°C, and the level of the acid-insoluble poly P fraction was measured as the amount of released Pi before and after hydrolysis of samples in the presence of 1 N HClO₄.

**Urea-PAGE.** Samples were applied to a 400-mesh carbon/Formvar-coated Cu grid, allowed to settle for 30 s, washed with two drops of water, and stained with uranyl acetate (1%) for 1 min. Samples were analyzed in a JEOL JEM-1230 transmission electron microscope at 80 kV; pictures were taken with a digital imaging camera (Gatan, Pleasanton, CA) at the Cell Imaging Facility, Beckman Center, Stanford University. Since *Synechococcus OS-B’* cells contain large amounts of condensed poly P granules, cells were starved of P for 2 weeks until no P granules were observed by staining with 4',6'-diamidino-2-phenylindole (DAPI). SEM. Cells were grown on 12-mm circular coverslips to late log phase and formed a biofilm-like structure. After the medium was removed (without drying out the cells), the samples were fixed in 2% glutaraldehyde and 4% paraformaldehyde (PFA) in 0.1 M cacodylate buffer, pH 7.3, at 4°C for at least 4 h and then allowed to settle onto polystyrene-coated 12-mm coverslips. After fixation, samples were washed briefly in 0.1 M sodium cacodylate and then incubated in 1% osmium tetroxide for 1 h at 4°C. After this incubation, the slides were dehydrated, gold-palladium coated, and visualized with a Hitachi S-3400N VP scanning electron microscope (SEM) (Cell Imaging Facility, Beckman Center, Stanford [http://tallow.stanford.edu/]). Cell length was quantified using Image J software. DAPI staining of poly P using confocal microscopy. Confocal microscope images were taken with a Leica SP5 AOBS point scanning spectral confocal microscope. Poly P granules were visualized by staining with 4',6'-diamidino-2-phenylindole (DAPI). Binding poly P to DAPI shifts its peak emission wavelength from 475 to 525 nm (excitation at 360 nm), allowing the use of DAPI for detection of poly P in vitro and in live cells. A long-wavelength excitation (≥400 nm) of the DAPI-poly P complex provides a dramatic increase in the sensitivity of poly P detection. Using excitation at 415 nm, as little as 25 ng/ml of poly P can be detected as cap and with a tiny hole at the bottom; the tube with disk was then placed inside a 1.5-ml Eppendorf tube. Poly P was eluted with 50 μl of elution buffer (10 mM Tris-HCl [pH 8.0], 500 mM KCl) by soaking the disk in the buffer for 10 min at room temperature and then centrifuging the tubes for 5 s at 14,000 rpm in a microcentrifuge; elution with 50 μl of buffer was repeated three times. To the combined 200 μl of eluate was added 2 μl of poly P (type poly P₇₀; 20 μg), and 6 μl of a 15% Norit suspension in water. The suspension was mixed and centrifuged for 5 min at 14,000 rpm in a microcentrifuge. The supernatant was transferred to a fresh Eppendorf tube, and the pellet was washed twice with 400 μl of 10 mM Tris-HCl buffer, pH 8.0. The supernatants were pooled to yield a total volume of 1.0 ml, which constituted the purified poly P extract. Protein levels were determined using bovine serum albumin as a standard (33). To estimate total poly P levels, we used the method of Ault-Riché et al. (31), in which the enzyme assay is performed with purified *Escherichia coli* polyphosphate kinase (Ec-PPK); in the presence of ADP and poly P, ATP is generated and measured using a luminescence assay as described by Ault-Riché and Kornberg (34).

**Electron microscopy.** Samples were applied to a 400-mesh carbon/Formvar-coated Cu grid, allowed to settle for 30 s, washed with two drops of water, and stained with uranyl acetate (1%) for 1 min. Samples were analyzed in a JEOL JEM-1230 transmission electron microscope at 80 kV; pictures were taken with a digital imaging camera (Gatan, Pleasanton, CA) at the Cell Imaging Facility, Beckman Center, Stanford University. Since *Synechococcus OS-B’* cells contain large amounts of condensed poly P granules, cells were starved of P for 2 weeks until no P granules were observed by staining with 4',6'-diamidino-2-phenylindole (DAPI). SEM. Cells were grown on 12-mm circular coverslips to late log phase and formed a biofilm-like structure. After the medium was removed (without drying out the cells), the samples were fixed in 2% glutaraldehyde and 4% paraformaldehyde (PFA) in 0.1 M cacodylate buffer, pH 7.3, at 4°C for at least 4 h and then allowed to settle onto polystyrene-coated 12-mm coverslips. After fixation, samples were washed briefly in 0.1 M sodium cacodylate and then incubated in 1% osmium tetroxide for 1 h at 4°C. After this incubation, the slides were dehydrated, gold-palladium coated, and visualized with a Hitachi S-3400N VP scanning electron microscope (SEM) (Cell Imaging Facility, Beckman Center, Stanford [http://tallow.stanford.edu/]). Cell length was quantified using Image J software. DAPI staining of poly P using confocal microscopy. Confocal microscope images were taken with a Leica SP5 AOBS point scanning spectral confocal microscope. Poly P granules were visualized by staining with 4',6'-diamidino-2-phenylindole (DAPI). Binding poly P to DAPI shifts its peak emission wavelength from 475 to 525 nm (excitation at 360 nm), allowing the use of DAPI for detection of poly P in vitro and in live cells. A long-wavelength excitation (≥400 nm) of the DAPI-poly P complex provides a dramatic increase in the sensitivity of poly P detection. Using excitation at 415 nm, as little as 25 ng/ml of poly P can be detected as...
TABLE 1 Primers used in this study

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<tr>
<th>Gene or primer name</th>
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<th>Reverse sequence</th>
<th>Purpose</th>
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a M, Y, and V are ambiguous bases that allow the primers to work for both Synechococcus OS-A and Synechococcus OS-B'. Y is T or C, M is A or C, and V is G or C.

fluorescence of the DAPI-poly P complex at 550 nm. Fluorescence emission from free DAPI and DAPI-DNA are minimal at this wavelength, making the DAPI-poly P signal specific and essentially independent of the presence of DNA (38). Western blot analysis. Following SDS-PAGE of cell extracts, the proteins were transferred onto nitrocellulose membranes using a Transblot Apparatus (Bio-Rad); the membranes were blocked with 5% (wt/vol) nonfat milk in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Polyclonal rabbit anti-Ec-PPK1 and anti-Pseudomonas aeruginosa polyphosphate kinase 1 (Pa-PPX1) antibodies were diluted (1:10,000) in PBS containing 0.05% Tween 20 before the addition of a 1:10,000 dilution of a goat anti-rabbit IgG peroxidase antibody conjugate in blocking buffer and incubated with blots at room temperature for 60 min. The blocking buffer was then washed three times for 20 min each with PBS containing 0.05% Tween 20 before the addition of a 1:10,000 dilution of a goat anti-rabbit IgG peroxidase antibody conjugate in blocking buffer for 30 min. Visualization of peroxidase-bound protein was performed using a Bio-Rad enzyme-linked immunosorbent assay (ELISA) kit.

Plasmid construction for transformation. A fragment of the Synechococcus OS-B' genome containing the ppk gene (CYB_2082) flanked by 400 bp of upstream and 300 bp of downstream sequence was amplified by PCR (Table 1 gives primer sequences) and cloned in pGemT (Promega). The sequences of the primers used for these analyses are shown in Table 1.

Transformation. Cells were grown at 45°C and bubbled with 3% CO2 until they reached log phase. Cells were washed once in 250 mM sucrose–8 mM HEPES (pH 7.4), resuspended at a concentration of 10^8 cells/ml, and then mixed with 20 µg of uncut plasmid DNA. The suspended cells were electroporated (Bio-Rad Gene-Pulser II electroporator) at 10 kV in a 0.1-cm cuvette. Following electroporation, the cells were resuspended in 1 ml of DH10 liquid medium supplemented with 250 mM sucrose and kept overnight in the shaker at 45°C in low light. Following the overnight treatment, cells were spread onto 0.5 µg/ml kanamycin-DH10 solid medium and incubated at 45°C for 12 months under low-light conditions (50 µmol of photons m^{-2} s^{-1}). Transforms appeared as small green colonies after ~15 days and were verified by using specific primers (Table 1).

In situ sample collection, extraction and RT-qPCR. Samples were collected on 28 and 29 July 2007 from the effluent channels of Octopus Spring at Yellowstone National Park (lat 44.5340, long −110.7978) (26). Further details about the location and geothermal features of Octopus Spring can be obtained at http://www.rcn.montana.edu/resources/features/. Frozen blast core samples or cells were processed for RNA or DNA extraction as described previously (25, 26). Briefly, samples for DNA extraction were collected using cork borers to remove an entire core/plug from the mat. Cores were separated (with a sterile blade) into the top green layer (typically, between 1 and 2 mm) and the bottom orange layer (typically, 2 to 4 mm). The top green layer contained the majority of the cyanobacteria and was used for nucleic acid extraction. Immediately after collection, samples were frozen in liquid nitrogen to prevent nucleic acid degradation.

RNA extraction. For analysis of transcript abundances, cells were harvested in log phase (~4 × 10^9 to 8 × 10^10 cells/ml). Total RNA extracted from the cells (25) was subjected to DNase I digestion (Turbo DNase; Ambion, Austin, TX), precipitated with ethanol, and tested by PCR for residual DNA contamination. Once the RNA was considered DNA free, it was either stored at −80°C or immediately used for reverse transcriptase quantitative PCR (RT-qPCR).

Reverse transcription and RT-qPCR. Reverse transcription of RNA samples was performed with 1 µg of DNA-free RNA, 200 ng of random hexamers (to prime the reverse transcription), 2 U of Superscript III RT (Invitrogen, Carlsbad, CA), the reaction buffer supplied by Invitrogen, and sterile, distilled water to a final reaction volume of 20 µl. The RT reaction was performed in a PTC thermocycler (MJ Research, Inc., St. Paul, MN) (12). Primers used in this study are listed in Table 1.

RESULTS

Visualization and estimation of poly P in Synechococcus OS-B’ cells. Synechococcus OS-B’ cells were grown in medium with phosphate (+P) until they reached stationary phase and then were stained with DAPI. Fluorescence microscopy indicated the presence of Poly P in Synechococcus OS-B’ cells.
ence of bright poly P granules, which were not visible in cells grown in medium lacking P (−P) (Fig. 1A). Although this particular protocol for visualization of the DAPI stain is fairly specific for poly P, it is a semiquantitative method (38). Therefore, we carried out analytical measurements of poly P present in Synechococcus OS-B’ in lag, logarithmic, and stationary phases, under +P or −P conditions. Synechococcus OS-B’ cells grown in +P medium accumulated high levels of poly P (49 ± 0.1 ng of poly P/mg protein) in stationary phase. In lag and log phase cells, the poly P levels were 5.1 and 9.8 ng of poly P/mg protein, respectively. Cells grown in medium lacking P had somewhat lower poly P levels, in the range of 3.5 and 5.2 ng of poly P/mg protein in lag and log phases, respectively (Table 2).

Based on assays in which the lengths of the poly P chains were estimated by urea-PAGE and standards of known size, it appears that Synechococcus OS-B’ cells contain high levels of relatively short-chain poly P (approximately in the range of P3 to P45), compared to E. coli, which has longer poly P chains (ranging from P10 to P750) (Fig. 1B). 31P nuclear magnetic resonance (NMR) analyses of Synechococcus OS-B’ cells grown in nutrient-replete medium also confirmed the accumulation of large amounts of poly P (Fig. 1C). To visualize poly P bodies by electron microscopy, Synechococcus OS-B’ cells were stained with 1% uranyl acetate and examined. The presence of four or five prominent darkly stained granules (or volutin granules) was noted in almost all cells (Fig. 2A and C). However, cells grown in medium lacking P were essentially devoid of these prominent poly P granules (Fig. 2B and D).

Identification of ppk and ppx genes. The complete genome sequences of Synechococcus OS-A and Synechococcus OS-B’ are available (11), which allowed us to perform BLAST searches using the well-characterized ppk gene from E. coli to identify the Synechococcus OS-B’ ortholog (CYB_2082). Synechococcus OS-A, a closely related cyanobacterium also contains a ppk gene, CYA_2477 (the amino acid identity [AAID] between these two PPK proteins is 94%). Both Synechococcus OS-A and Synechococcus OS-B’ PPK1 contains the 11 conserved residues essential for a presumed poly P tunnel (see Fig. S1 in the supplemental material). Based on comparative analysis, Synechococcus OS-B’ PPK appears to have a 40-amino-acid extension at the N terminus that is missing in Synechococcus OS-A and in other cyanobacteria; based on the amino acid sequence, it does not have characteristics of a signal sequence, so its role is currently unknown.

The ppx gene encoding the exopolyphosphatase PPX, responsible for the productive degradation of poly P, was also identified in the genome of both hot spring cyanobacteria (CYB_1493 and CYA_2432; 92% AAID). We also searched the metagenome database derived from the same microbial mat from which Synechococcus OS-A and OS-B’ were originally isolated and identified numerous ppx and ppk genes (11). Based on homology, some of these genes were identified as being from Synechococcus sp. while others were identified as putatively belonging to the filamentous anoxygenic phototrophs (FAPs) Roseiflexus and Chloroflexus spp., which are prominent members of the mat community (data not shown) (40). These FAPs also contain other enzymes associated with poly P metabolism, including the poly P-AMP-phosphotransferase (41). There are no genes encoding obvious homologs of PPK2 (42), the GTP generator, on the Synechococcus OS-A or OS-B’ genome, but Chloroflexus sp. strain Y-400–fl possesses a gene encoding a putative PPK2 (YP_002571783.1). Thus, it ap-

![Image](http://jb.asm.org/)
pears that the cyanobacteria and FAPs, which are the dominant photosynthetic organisms in these microbial mats, contain a complement of proteins that allow them to store and utilize poly P.

**Transformation of Synechococcus OS-B’ cells.** To assess the role of poly P in the growth and survival of *Synechococcus* OS-B’, we created mutants in which the *ppk* gene was disrupted with a thermostable kanamycin cassette (39, 43). Since these organisms have only recently been grown as isolates under laboratory conditions, we had to first optimize conditions for transformation.

To transform isolates of *Synechococcus* OS-B’ cells, we first determined that the isolate was sensitive to several commonly used antibiotics at low levels (between 0.1 and 2 μg/ml) (data not shown). Next, we constructed a plasmid in which the *ppk* gene was disrupted by the kanamycin cassette obtained from the plasmid pT7BT/P*cpcC::Km* (39). This plasmid was used for subsequent transformation experiments. We initially attempted “natural transformation”; i.e., plasmid DNA was incubated directly with untreated cells since this has been used successfully to transform cyanobacteria (39), but this method had a very low success rate and was not reproducible. Attempts to starve cells (by growing in medium lacking N or P or by long incubations in the dark) to reduce the large amount of extracellular exopolysaccharides (which could inhibit transformation) did not appreciably increase the transformation frequency. However, we found that using electroporation with large amounts of DNA (~20 μg of DNA per transformation) (see Materials and Methods) produced the most consistent results. We have not tried an exhaustive set of conditions, so it is possible that in future experiments we may be able to enhance rates of transformation using other protocols. However, for the purpose of this analysis, we were able to create the requisite *ppk* mutant strain. PCR analysis showed that the *ppk* gene had been disrupted by integration of the kanamycin cassette into the chromosome by a double homologous recombination event (Fig. 3).

**Growth of *ppk* mutant cells under different conditions and expression of Pho regulon genes.** *Synechococcus* OS-B’ and *ppk* cells grew at similar rates in both P-replete medium and in medium lacking P and bubbled with 3% CO₂ (Fig. 4A). Cells grown under replete conditions undergo at least five doubling times before reaching stationary phase, while under −P conditions, both wild-type (WT) and *ppk* mutant strains do not seem to grow. Despite similar growth rates, there was a noticeable difference in

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**FIG 2** Electron microscopy images of *Synechococcus* OS-B’ and *ppk* mutant cells. Cells of WT *Synechococcus* OS-B’ (A and C) and the *ppk* mutant (B and D) stained with uranyl acetate (1%) are shown. Images in panels C and D are at low magnification, while images in panels A and B are at high magnification. Note the darkly stained volutin granules.
the levels of transcripts from the various genes that are part of the Pho regulon in WT and ppk mutant cells during nutrient-replete growth. Transcript levels of phoR (CYB_0858) and phoB (CYB_2856), the genes that encode the two-component regulatory system of the Pho regulon, were upregulated 10- to 12-fold in the ppk mutant relative to WT cells when both strains were grown in P-replete medium. Genes encoding two phosphatases, ppx (CYB_1988), the phosphate transporter (pscC-2, CYB_1916), and a ftsY operon (ftscC-1, CYB_0159) were upregulated from 50- to 150-fold in P-replete medium. Genes encoding two phosphatases, ppk (CYB_1916), and a (ppkF); lanes 8 and 9, primers in the ppk gene. (B) The expected sizes are 999 bp for the WT strain, 849 bp for KanF+R, and 1,598 bp for ppkF+R, and 1,522 bp for the ppkΔkan strain.

FIG 3 Identification of ppk transformant. (A) PCR result showing the insertion of the kanamycin cassette (in the reverse direction) within the ppk gene. Lane 1, GeneRuler 1-kb DNA ladder. Other lanes show results with PCR products from reactions using Synechococcus OS-B’-DNA (lanes 2, 4, 6, and 8) or using DNA of ppk transformants (lanes 3, 5, 7, and 9). Primers A0017F and A0017R (A0017F+R, where F and R indicate forward and reverse, respectively) were used as positive controls for DNA quality (lanes 2 and 3). Other primers used were as follows: lanes 4 and 5, primers in the galactose cassette; lanes 6 and 7, primers in the kanamycin cassette (KanF) and in the ppk gene (ppkF); lanes 8 and 9, primers in the ppk gene. (B) The expected sizes are 999 bp for the WT strain, 849 bp for KanF+R, and 1,598 bp for ppkF+R, and 1,522 bp for the ppkΔkan strain.

Next, we examined growth rates of cells under two different conditions: cultures were bubbled with air or with CO2-enriched air (3% CO2). WT cells grew at the same rates when bubbled with either air or 3% CO2, but the ppk mutant cells showed impaired growth when maintained in air; the cells divided only three times (Fig. 4B). This unexpected result suggested a possible link between the activity of the carboxysome which is required for efficient CO2 uptake and P metabolism. To explore this further, we analyzed levels of transcripts encoding proteins required for carboxysome biogenesis in both WT and ppk mutant cells and found that in mutant cells the transcript levels from three genes (CYB_1794, ccmM; CYB_1795, ccmL; and CYB_1796, ccmK1) encoding components of the carboxysome were elevated from 50- to 150-fold (Fig. 5). However, the first gene in this cluster, CYB_1797 (ccmK2) did not exhibit an equivalent increase. Further analysis will be required to understand the link between poly P levels and carboxysome synthesis and function.

**Phenotype of ppk mutant cells.** ppk mutant cells are defective in synthesis and accumulation of poly P granules; ppk mutant cells grown in P-replete medium contained only 2% of the levels of poly P (~0.8 ± 0.1 ng/mg protein) compared to WT Synechococcus OS-B’ cells (~50 ng/mg protein). This result was corroborated by electron micrographs of the mutant stained with uranyl acetate. WT cells showed large poly P bodies, as described earlier (Fig. 2A and B) while the ppk mutant lacked visible poly P granules (Fig. 2C and D).

Reduced EPS accumulation and reduced size of ppk cells. Scanning electron microscopy (SEM) of Synechococcus OS-B’ and the ppk mutant treated with OsO4 (Fig. 6) indicated that the ppk cells had two morphologically identifiable phenotypes. First, the amount of secreted exopolysaccharide (EPS) was diminished in the ppk mutant, as visualized by SEM. In contrast, WT cells had large amounts of EPS material which could be visualized outside the cells and on the grid surface. Second, the ppk mutant appeared, on average, to be approximately 40% shorter than WT cells (Synechococcus OS-B’ cells on average are 10.3 ± 1 μm long, and ppk mutant cells are 6 ± 1 μm). The shape and size of WT cells appear to be affected by growth conditions (Devaki Bhaya, unpublished observations); thus, without a more detailed analysis, the exact cause of the smaller ppk cells is not clear. However, the finding that PPK can form tubular structures suggests a potential role of PPK in the maintenance of cell shape (27, 44) (see the Discussion; also see Fig. S2 in the supplemental material). The morphological changes associated with the ppk mutant are consistent with reports that EPS production is decreased in ppk mutants of pathogenic bacteria (45) and that a polyphosphate kinase 1 (ppk1) mutant of *Pseudomonas aeruginosa* exhibits multiple ultrastructural and functional defects.

**In situ levels of ppk and ppx transcripts and PPK and PPX.** To understand the role played by poly P in mat-dwelling organisms, we measured the levels of ppk and ppx transcripts in RNA extracted from mat samples collected at Octopus Spring during different times of the diel cycle. Oligonucleotides were designed to specifically amplify conserved regions of the ppk and ppx genes from *Synechococcus OS-B’* and *Synechococcus OS-A* (Table 1). The ppk transcript is at its maximal level during the evening; on the other hand, ppx transcript amounts are highest at the start of the daylight period (Fig. 7, lower panel). To further consolidate this interesting observation, we measured PPK and PPX levels in the mat by Western blotting using specific antibodies generated against Ec-PPK1 and Pa-PPX, respectively (Fig. 7, upper panel). We observed that PPK levels were highest in the late evening, suggesting that the poly P accumulated during the night, while PPX levels were high in the early morning. These data are consistent with the transcript analysis, and the same trend is observed with *Synechococcus OS-B*’ isolates (see Fig. S3 in the supplemental material). We hypothesize that during the dark period when photosynthesis is not occurring, growth is reduced, and available P is stored as poly P. This poly P may serve as a form of readily available energy during the early morning when not enough ATP is being produced by photosynthesis.

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DISCUSSION

Role of Poly P in cyanobacteria. It has been suggested that inorganic polyphosphate was present in prebiotic environments and is proposed to be an “ancient” molecule (46, 47), which also plays an important biological role. For instance, stationary-phase cells of Vibrio cholerae, Salmonella spp., or E. coli contain high poly P levels, ranging from 20 to 40 ng of poly P/mg of protein under stress conditions (20). Studies of poly P in these microbes suggest that it plays a multifunctional role in regulating stress responses, with mutants showing pleiotropic effects (36, 48). Less is known about the importance of poly P in cyanobacteria; here, we have analyzed the role of poly P in a thermophilic cyanobacterium from microbial mats and its role in survival in harsh environments.

Poly P granules were observed in the cyanobacterium Ana- cystis nidulans (Synechococcus elongatus PCC 7942) under conditions of sulfur deficiency (49, 50), and in other cyanobacterial species, poly P levels of up to 20 nmol/mg of protein have been measured in stationary-phase cells (21). By comparison, Synechococcus OS-B’ accumulates very high levels of poly P (~50 nmol of poly P/mg of protein) under conditions when P is available, and this may reflect adaptation to extreme environments such as the hot springs which are generally resource poor. The genomes of FAPs (e.g., Chloroflexus sp. Y-400-fl and Roseiflexus sp.), which are another major group of organisms in microbial mats, contain high poly P levels as well, and this may reflect common evolutionary pathways.

FIG 4 Growth of Synechococcus (Syn) OS-B’ and ppk cells. (A) Growth of Synechococcus OS-B’ WT and ppk mutant (ppk) cells at 50°C for 72 h in complete medium or in medium devoid of P and bubbled with 3% CO2-enriched air. (B) Growth of Synechococcus OS-B’ WT and the ppk mutant in medium bubbled with 3% CO2-enriched air and air alone. For panels A and B the numbers of cells per ml are shown on the y axis on a log scale. (C) Quantification of transcripts (relative units, RU) encoding PhoR (CYB_0858), PhoB (CYB_2856), PPX (CYB_1493), PhoX(CYB_1988), PstC-2 (CYB_1916), and PhnC-1 (CYB_0159) in Synechoccus OS-B’ and ppk mutant cells.

FIG 5 Quantification of transcripts encoding ccmM, ccmL, and ccmK1 in Synechococcus OS-B’ and ppk cells and genomic organization of the ccm operon in Synechococcus OS-B’. Quantification of transcripts encoding CYB_1794 (ccmM), CYB_1795 (ccmL), and CYB_1796 (ccmK1) in Synechococcus OS-B’ (black bars) and ppk cells (white bars). RT-qPCR results (relative units, RU) show the mean and standard deviations for data from three technical replicates although similar results were obtained for biological replicates. Cells were analyzed after 72 h of growth though similar results were obtained at stationary phase.
mats, also encode polyphosphate kinases and exopolyphosphatases (unpublished observations). We also found evidence for a polyphosphate kinase 2 (PPK2, YP_002571783.1), which is able to generate GTP from poly P; however, neither *Synechococcus* OS-A nor OS-B′ encodes an obvious polyphosphate kinase 2. Thus, it appears that cyanobacteria and FAPs, which are the dominant photosynthetic organisms in these microbial mats, are capable of synthesis, degradation, and utilization of poly P. In a preliminary examination of cells taken directly from microbial mats and stained with DAPI, we found evi-

![FIG 6](image6.png) Scanning electron microscopy images of *Synechococcus* OS-B′ (WT) and ppk cells (M). (A and C) *Synechococcus* OS-B′ cells attached to a surface. (B and D) ppk cells. On average, *Synechococcus* OS-B′ cells are somewhat longer than ppk mutant cells and have a higher level of EPS surrounding them.

![FIG 7](image7.png) Transcript levels of ppk and ppx and protein levels of PPK and PPX in mat samples over a diel cycle. (Top) Western blot analyses of PPK and PPX. PPK and PPX were detected in protein extracts from mat samples collected at various times during the diel cycle. Both proteins were detected using antibodies generated against *E. coli* PPK (anti-Ec-PPK) or *Pseudomonas aeruginosa* PPX (anti-Pa-PPX). (Bottom) Quantification of transcripts from ppk and ppx over the diel cycle. Transcript levels are expressed as relative units (RU).
dence for considerable amounts of poly P (unpublished observations) although at this point we do not know how much these levels fluctuate temporally or seasonally or if there is a high degree of cell-to-cell variability in poly P levels within mat samples.

In situ transcriptomics of ppk and ppx. In situ transcriptomics and protein level estimation clearly demonstrate that enzymes responsible for the synthesis and degradation of poly P undergo diurnal oscillations, with maximal levels occurring at distinct times of day over the diel cycle. These studies suggest that the storage and use of poly P in thermophilic cyanobacteria are a dynamic process that may, in turn, reflect the energetic status of the cell. During the evening and night, when photosynthesis stops, growth is reduced, and the requirement for ATP is consequently diminished. At this point in the diel cycle, PPK1 could function to convert available P into poly P as a storage form of energy. Conversely, in the early morning when not enough ATP is being generated from photosynthesis, this poly P storage form could serve as a ready and supplementary source of ATP. The pattern of accumulation of the proteins for synthesis and breakdown of poly P (Fig. 7) would suggest that at night, when the net use of P is reduced, the cells store incoming P as poly P (PPK levels are high), while in the early morning, PPX breaks down stored poly P. Such a scenario is also consistent with the diurnal changes observed in other pathways, such as respiration and fermentation (25, 26).

In earlier investigations, in situ transcriptomic data on mat samples indicated the existence of large diurnal fluctuations in transcripts of enzymes required for fermentation, respiration, photosynthesis, oxidative stress, and nitrogen fixation (25–27). These results were also corroborated by functional assays of photosynthesis and nitrogen fixation (26) and point to the existence of metabolic windows of opportunity such that certain functions occur at particular times of the diel cycle. The optimization of these processes may depend on several factors, such as energetic requirements, metabolic status of the cell, gene regulation, and posttranscriptional effects.

Over and above changes in requirements for stored P for optimal growth of cells that occur on a diel cycle, there may also be long-term conditions when P is unavailable or is in excess (the so-called "feast-or-famine" situation). When P is in excess, either in the mat environment or under defined laboratory culture conditions, cells store poly P; conversely, under nutrient deprivation or famine conditions, the poly P store is a potential source of P for the energetic requirements of the cell and may also allow the sustenance of the cells under stressful conditions. In resource-poor environments, such as the microbial mats, the ability to scavenge, store, and utilize P may be an important survival strategy. The opposing activities of the two enzymes polyphosphate kinase and polyphosphatase could allow cells to store and utilize poly P to optimize growth over a diel cycle or to maintain optimal growth over longer time scales. Furthermore, this trend can be reproduced under defined laboratory conditions, suggesting that the regulatory aspects of this control can be further studied with isolates. Indeed, ppk mutant cells have a very small amount of poly P, and a number of phenotypes that are either directly or indirectly a consequence of these low-poly P levels are exhibited by these cells.

Phenotypes of the ppk mutant. The pleiotropic effects of inactivating the ppk gene suggest that poly P not only serves as a source of stored energy but may also help the cell adapt to other stress conditions. This parallels the findings of other groups working with pathogenic and nonphotosynthetic bacteria where ppk mutants are compromised in their ability to cope with stress (45). For instance, in this study we show that ppk mutant cells do not grow well under nutrient-depleted conditions, particularly if they are grown in air rather than in CO2-enriched air. Although the exact cause of this growth impairment phenotype will require additional experimentation, the lack of growth in air might be the consequence of impaired CO2 fixation at air levels of CO2 resulting from a shortage of energy related to the lack of poly P. This suggests an intriguing link between the poly P storage function and photosynthesis and/or carbon fixation which is yet to be fully understood. Of the four ccm genes examined in this study, all of which are involved in carboxysome biosynthesis, three (ccmK1, ccmL, and ccmM) showed strong upregulation in the ppk mutant. This may indicate that when poly P content in the cell is reduced, carboxysomes are not assembled efficiently, which explains why the mutant cells grow better in air enriched with CO2, where there is a reduced requirement for an active carbon-concentrating mechanism via carboxysomes.

In this context, there have been a few recent studies linking poly P granules and carboxysome function. Cryo-electron microscopic studies have demonstrated that carboxysomes generally cluster into distinct groups within the cytoplasm, often in the immediate vicinity of poly P granules. Furthermore, a regular lattice or strings frequently connect poly P granules to nearby carboxysomes (27); while it is unclear what constitutes these lattices and strings, it is suggested that they may be oligomers of PPK. This is consistent with the observation that, in the presence of ATP, filaments have been observed in purified samples of PPK1 from Pseudomonas aeruginosa (see Fig. S2 in the supplemental material) and of PPK2 samples from Dictyostelium discoideum (37). Carboxysomes appear to be spatially organized in Synechococcus sp. strain PCC7942 cells (51), and the images showing their position within the cell are similar to the ones showing poly P granules in Synechococcus OS-B’. The internal organization of the bacterial cell is important for the efficient functioning of biosynthetic and metabolic pathways (52), and several recent reports indicate that bacteria contain filaments and microtubular cytoskeletal networks responsible for the size and shape of the cell, as well as for the formation of discrete microcompartments (53, 54). MreB and ParM, for example, both engage in the formation of actin-like filaments that are indispensable in the maintenance of cell shape and plasmid segregation (55, 56). Poly P is known to have a role in maintaining the extended shape of the nucleoid and the cell envelope so that in Pseudomonas aeruginosa ppk1 mutant cells, the nucleoid is compacted, and the cytoplasm is detached in many places from the cell poles and borders (44). Other proteins like CTPase (37, 58) have also been reported to make filaments inside the cell. The fact that ppk mutant cells appear to be shorter than WT cells suggests that there may be a spatial role associated with the presence of poly P in the cell, but this role remains to be explored in Synechococcus OS-B’.

The transformation system developed for Synechococcus OS-B’ has allowed us, for the first time, to investigate the role of poly P on growth and survival in a photosynthetic microorganism. In many other nonphotosynthetic bacteria including pathogens, it has been shown that poly P plays a number of roles ranging from survival under stationary phase to motility and virulence (20). This study, which combines the characterization of a ppk mutant and also investigates levels of ppk and ppx transcripts and protein...
in situ, opens the door for further examination of how cyanobacteria cope with fluctuations in phosphorus, one of the nutrients that has a major impact on the biogeochemistry of both terrestrial and marine environments.

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