

Phosphorus Deprivation Responses and Phosphonate Utilization in a Thermophilic *Synechococcus* sp. from Microbial Mats^{∇†}

Melissa M. Adams,^{1,2} María R. Gómez-García,¹ Arthur R. Grossman,¹ and Devaki Bhaya^{1*}

Carnegie Institution for Science, Department of Plant Biology, Stanford University, Stanford, California 94305,¹ and Department of Biology, Stanford University, Stanford, California 94305²

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The genomes of two closely related thermophilic cyanobacterial isolates, designated *Synechococcus* isolate OS-A and *Synechococcus* isolate OS-B', from the microbial mats of Octopus Spring (Yellowstone National Park) have been sequenced. An extensive suite of genes that are controlled by phosphate levels constitute the putative Pho regulon in these cyanobacteria. We examined physiological responses of an axenic OS-B' isolate as well as transcript abundances of Pho regulon genes as the cells acclimated to phosphorus-limiting conditions. Upon imposition of phosphorus deprivation, OS-B' stopped dividing after three to four doublings, and absorbance spectra measurements indicated that the cells had lost most of their phycobiliproteins and chlorophyll *a*. Alkaline phosphatase activity peaked and remained high after 48 h of phosphorus starvation, and there was an accumulation of transcripts from putative Pho regulon genes. Interestingly, the genome of *Synechococcus* isolate OS-B' harbors a cluster of *phn* genes that are not present in OS-A isolates. The proteins encoded by the *phn* genes function in the transport and metabolism of phosphonates, which could serve as an alternative phosphorus source when exogenous phosphate is low. The *phn* genes were upregulated within a day of eliminating the source of phosphate from the medium. However, the ability of OS-B' to utilize methylphosphonate as a sole phosphorus source occurred only after an extensive period of exposure to the substrate. Once acclimated, the cells grew rapidly in fresh medium with methylphosphonate as the only source of phosphorus. The possible implications of these results are discussed with respect to the ecophysiology of the microbial mats.

Significant advances have been made in characterizing the diversity and ecophysiology of microorganisms in the alkaline hot spring microbial mats of Yellowstone National Park (4, 53). However, relatively little is known about specific physiological adaptations and the acclimation responses of microbes in the mats with respect to nutrient limitation (1, 40, 42, 43). A unicellular cyanobacterium, *Synechococcus* sp., is the dominant, primary producer in these hot spring microbial mats at temperatures above 50°C and is found exclusively in the top green layer (comprising 1 to 2 mm of the ~1.5-cm-thick mat) (53). Based on denaturing gradient gel electrophoresis analysis and the subsequent determination of the 16S rRNA/internal transcribed spacer sequence, it appears that *Synechococcus* sp. ecotypes are delineated along environmental gradients, including temperature and light (9, 10, 37). For instance, the *Synechococcus* OS-A isolate (hereafter OS-A) is abundant in the high-temperature region of the mat (58 to 65°C), while the OS-B' isolate is abundant in the lower-temperature regions (50 to 60°C). OS indicates that the organisms were isolated from Octopus Spring (9).

We have complete genome sequences for *Synechococcus* OS-A and OS-B' isolates, which have enabled examination of the genetic potential of these organisms to respond to fluctuating environmental conditions, such as nutrient levels, light intensities, and oxygen tension (3, 42, 43). Recently, we gen-

erated an axenic culture of OS-B' and investigated acclimation responses to light intensities that they would experience in the microbial mat (19). It was found that OS-B' cultures grew best at relatively low light intensities (25 to 200 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$), despite the high irradiation levels experienced by the cells in the natural environment (43). In fact, relatively high light elicited several acclimation responses, including chlorosis or cell bleaching, which reflects a reduction in the levels of chlorophyll *a* (*chl a*) and phycobiliprotein (PBP).

We are studying the ways in which axenic cultures of thermophilic cyanobacteria respond to a variety of defined environmentally relevant conditions in the laboratory. We have initiated such studies by defining the potential gene members of the Pho regulon and characterizing accumulation of transcripts from these genes in response to P deprivation. The levels of inorganic phosphate (P_i) in the effluent channel of OS have been determined to be as low as 0.37 μM (33), although much higher levels (17 μM) have also been reported (4). As a reference, P starvation responses of *Escherichia coli* are elicited when the extracellular P_i concentrations drops below 4 μM (49, 51). The dominant P source within the hot spring microbial mat is P_i , as determined by ³¹P-nuclear magnetic resonance analysis of the total P content (M. Adams and B. Cade-Menun, unpublished data); other P compounds detected include phosphonates and polyphosphate. Based on the ³¹P-nuclear magnetic resonance analysis, phosphonates could constitute up to 5% of the total P content in the microbial mat, and levels may fluctuate within the microbial mat over the diel cycle. However, the P composition of the aqueous environment surrounding the microbial mat (extracellular) was not investigated.

P_i metabolism has been studied extensively in *E. coli* (46,

* Corresponding author. Mailing address: Carnegie Institution for Science, 260 Panama Street, Stanford, CA 94305. Phone: (650) 325-1521. Fax: (650) 325-6857. E-mail: dbhaya@stanford.edu.

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49). The levels of over 80 identified proteins increase in response to P starvation, and many of these proteins are encoded by genes that are coregulated as part of the Pho regulon (47, 49). The multigene transcriptional induction associated with the Pho regulon is accomplished by a mechanism in which the transcriptional regulator PhoB binds to the Pho box sequence, which is an upstream activation sequence that precedes each operon of the Pho regulon (25, 49). Many of the transcriptional units that are part of the Pho regulon represent multigenic operons, such as the multigenic transport operon that encodes the four subunits (PstS, PstC, PstA, and PstB) of the Pst high-affinity P_i transport systems (25, 49). The PstSCAB transporter operon in bacteria often includes the gene encoding PhoU, which is thought to function as a negative regulator of the PhoR-PhoB two-component system (31). Genes of the Pho regulon also encode phosphatases (e.g., PhoA), which liberate P_i from organic sources in the environment, and the enzymes required for utilization of other P sources, including UGP-glycerol phosphate (41). The Pho regulon in *Pseudomonas fluorescens* Pf0-1 lacks a PhoA homolog but encodes a phosphatase, PhoX, which is predominantly responsible for extracellular phosphatase activity during P limitation (29). Secretion of PhoX by the twin-arginine transport pathway is a feature that distinguishes this family of phosphatases from the PhoA family (17).

The enzymes polyphosphate kinase 1 (PPK1) and exopolyphosphatase (PPX) have been shown to be members of the Pho regulon of *E. coli* K-12 (GenBank accession numbers NP_416996 and NP_416997 for PPK1 and PPX, respectively) (18) and are also present in the model cyanobacterium *Synechocystis* strain PCC 6803 (accession numbers NP_442590 [sll0290] and NP_442969 [sll1546] for PPK1 and PPX, respectively) (11). PPK1 is a highly conserved enzyme in prokaryotes, found in over 100 bacterial genomes to date, and is responsible for the reversible polymerization of ATP to make polyphosphate [poly(P)] (5). PPX and other enzymes with PPX activity, such as the periplasmic acid phosphatase SurE (36) or the guanosine pentaphosphate phosphohydrolase (18), degrade poly(P) in prokaryotes under conditions of P starvation.

Currently, the only well-studied cyanobacterial Pho regulon is that of *Synechocystis* strain PCC 6803, which features a sensor histidine kinase (SphS) and response regulator (SphR) that are orthologs of PhoR and PhoB, respectively, of *E. coli* (13). Three operons have been identified that are activated by SphR in *Synechocystis* strain PCC 6803, including two ABC-type high-affinity P_i uptake systems (*pstS1-C1-A1-B1-B1* and *pstS2-C2-A2-B2*) and *phoA-nucH*, encoding an alkaline phosphatase and extracellular nuclease (45). Recently, in silico predictions of Pho box elements upstream of cyanobacterial genes have been performed for 19 different cyanobacteria that have fully sequenced genomes, including *Synechococcus* OS-A and OS-B' isolates (44).

Polypeptides involved in the uptake and assimilation of P from molecules containing a phosphonate (Phn) bond are also part of the Pho regulon of *E. coli* (20, 27, 54). The proteins required for Phn utilization include transport components (PhnCDE) and a specific multisubunit C-P lyase (Phn-FGHIJ KLMNOP) that is required for hydrolysis of the C-P bond (27). The C-P bond of Phns is recalcitrant to chemical hydrolysis, thermal denaturation, and photolysis, making these com-

pounds markedly different from other P sources (32). Although Phns are found in organisms across phylogenetic groupings, only prokaryotic microorganisms have, so far, been shown to have the capability to cleave the Phn bond. Phns are ubiquitous and have been identified in eukaryotes, bacteria, and archaea as antibiotics and phosphonolipids, although the exact role of the latter within the lipid bilayer is still not clear (54). The most common naturally occurring Phn is aminoethylphosphonate (AEPPhn), which is present in phosphonolipid head groups as well as in polysaccharide and glycoprotein side groups (54). In the marine environment, Phns can comprise as much as 25% of the high-molecular-weight dissolved organic P and serve as a P source for some bacteria (7).

Many Phns found in terrestrial and aquatic environments are anthropogenic in origin, as they are components of flame retardants, plasticizers, pharmaceuticals, and herbicides. Methylphosphonate is a component of several synthetic compounds, such as the flame extinguisher Pyrol 67, which is composed of vinylphosphonate and methylphosphonate. Glyphosate [*N*-(phosphonomethyl)glycine] is the active ingredient in the widely used agricultural herbicide Roundup (26). There are potential repercussions of the widespread use of Phns as herbicides, since some are potentially toxic and have long retention times in the environment (20).

In this study, we examined the physiological consequences of P deprivation on axenic isolates of the thermophilic cyanobacterium isolate *Synechococcus* OS-B'. We used a number of different methods, including assays for alkaline phosphatase activity, quantification of transcripts of the Pho regulon, and monitoring of cell doubling time, to assess the ability of OS-B' to grow on Phn as the sole source of P. Under P deprivation conditions, the cells bleached and developed elevated levels of alkaline phosphatase (APase) activity. Transcripts from the putative Pho regulon genes (including *phoA*, *phoD*, *phoX*, and *pstS*) accumulated in cells soon after they were transferred to medium devoid of P. Furthermore, genes involved in the utilization of Phns were expressed in P-starved OS-B' cells. These results are discussed in the context of cyanobacterial ecophysiology in hot spring microbial mats.

MATERIALS AND METHODS

Culture conditions. The original *Synechococcus* isolate from Octopus Spring was designated JA-2-3B'a (2-13) (1). After generating an axenic culture of the strain, we designated the isolate *Synechococcus* sp. OS-B' (CIW-10), which was grown and maintained at 55°C in liquid D medium (6) supplemented with 10 mM HEPES (pH 8.2 to 8.3) and Va vitamins (2). This growth medium, termed DH10, contains P_i as sodium phosphate at a concentration of 0.77 mM. For preparation of P_i -free medium, the sodium phosphate was replaced with an equimolar amount of sodium chloride. Cultures were bubbled with a mixture of 3% CO_2 in air, in a 55°C incubator, and under continuous, relatively low light ($\sim 75 \mu\text{mol photon m}^{-2} \text{s}^{-1}$). The irradiation was measured with a Li-Cor LI-189 quantum meter using a Li-Cor LI-193SA spherical sensor.

Growth measurements. Growth rates and whole-cell absorbance spectra for OS-B' were measured following growth in P-replete (+P) or P-free (-P) medium. Culture aliquots were collected at the same time every day; cell densities were estimated by counting cells using an Ultraplane Neubauer hemocytometer (Hausser Scientific, Pittsburgh, PA) and monitoring optical density of the culture at 750 nm (OD_{750}) to generate a correlation between the OD_{750} and cell number. The OD_{750} showed a linear correlation with cell number over the first 200 h of growth in either +P or -P medium. Despite the characteristic loss of photosynthetic pigments during growth of OS-B' under P starvation conditions, the relationship between cell density and OD_{750} remained relatively constant into stationary phase of the P-starved cells. OS-B' cultures were started at an OD_{750}

TABLE 1. Primers used to quantify putative Pho regulon transcripts of OS-B'^a

Gene	Locus tag	Size		Forward primer	Reverse primer
		nt	aa		
phoA	CYB_1198	1,349	449	TGGTGC AAACGGGATCCATCATTG	AATCTCCTCATAGTCGCTGCGCTT
phoD	CYB_0684	1,727	575	GCCGCGGCGATATCGATTTTCATTT	CAACAAAGGAAGTCCGGCCAAAACA
phoX	CYB_1988	2,048	682	ACGATGCCCGCTTTGAGTACATCT	CTTGGCCACATACAAGGTGCCATT
pstS-1	CYB_1077	1,076	358	CACAGGTGACTTCCCGAAT	ATTCCACGTAGCCAATCGAG
pstS-2	CYB_1915	1,061	353	ATGCGAACCTGCTTTCTGCTTTC	GAGATTTGCACGGTTTGGGCTTGA
phnC-1	CYB_0159	791	263	AAACAAGGTTGCCCTAAGGGAGGT	TGGCCTTCATGGAGAGGAAGAGAA
phnD-1	CYB_0160	896	298	GCTCCATTGAAGCGTTCGTGAAA	TTGCCCTTGGCGTCTTCTAGAGTT
phnC-2	CYB_1467	809	269	TGGCTCAATGGAATCGACCTCACT	TAGCCCAACTGACCCGAAAGAACA
phnI	CYB_0164	696	231	TGCTGGATTGGAATGGATCGCC	AGTGGCTATCCAGTGTCTGAGAA
phnJ	CYB_0165	866	288	GTAGCATATGAAACCAAGCATAG	AACTCGAGAGGACCGTCTCGTTT

^a Included in the table are locus tags, the sizes of the gene in nucleotides (nt) and amino acids (aa), and both the forward and reverse primer sequences that were used.

of 0.025 (~10⁶ cells/ml) and grown to late log phase (OD₇₅₀ of 0.25 to 0.4, or ~10⁷ cells/ml). Cells were washed twice in DH10-P medium to remove traces of P_i and then resuspended in fresh medium (either +P or -P) to an OD₇₅₀ of 0.025 (~10⁶ cells/ml). The growth of OS-B' was monitored into stationary phase, and the experiment was terminated at 192 h. All growth experiments were repeated at least three times.

Growth on MePhn. The same methods were used to monitor growth of OS-B' cells after they were transferred to +P or -P medium supplemented with methylphosphonate (MePhn), which was added to the medium by sterile filtration to a final concentration of 0.5 mM. This concentration of Phn was chosen because previous studies established that it supports optimal growth of *E. coli*, *Pseudomonas stutzeri*, and *Rhizobium meliloti* (34, 52). Other C-P compounds were also assessed as potential P sources, including ethylphosphonate (EthPhn), iN-(phosphonomethyl) glycine, AEPPhn, and phosphonomycin. Of these, only EthPhn (like MePhn) could support the growth of OS-B' as a sole source of P.

Cell viability. Cell viability was monitored with the Live/Dead BacLight kit L-7012 (Molecular Probes, Inc., Eugene, OR), as previously described (24).

Alkaline phosphatase activity. APase activity was measured at 24-h intervals over the course of an experiment by using a colorimetric assay modified from the methods of Ray et al. (39). Briefly, extracellular APase activity was assayed over the course of 15 min. A 500- μ l aliquot of harvested cells at each respective time point was added to an equal amount of the colorimetric reagent *p*-nitrophenyl phosphate (pNPP) in Tris buffer, generating a reaction mix containing 3.6 mM pNPP with 200 mM Tris HCl, pH 7.0 to 9.0. Cells were removed by centrifugation, and the absorption of the supernatant was measured at 410 nm. APase activity was calculated as μ g pNPP hydrolyzed per h per 1 \times 10⁶ cells.

Spectral data. For each time point in the growth analyses, whole-cell absorbance spectra from 350 to 800 nm were measured to determine relative chl *a* and PBP contents (8). The spectra were normalized to the OD₇₅₀ of the culture, which allowed comparisons of spectral features across different cell densities, since the OD₇₅₀ could be used as a proxy for cell number under all growth conditions.

RNA extraction. For analysis of transcript levels from putative Pho regulon genes over the time course of growth and under different P conditions (+P, -P, +P+MePhn, and -P+MePhn), cells were harvested at 24-h intervals throughout the logarithmic phase of growth (24, 48, 72, and 96 h). For experiments in which P_i was added to cultures following 72 h of growth in +P or -P medium, the P_i was filter sterilized and added to a concentration of 0.77 mM (if the concentration in the culture after the initial growth period was not considered). RNA was extracted from cells as previously described (42). Isolated RNA was subjected to DNase digestion (Turbo DNase; Ambion, Austin, TX), precipitated with ethanol, and tested by PCR for residual DNA contamination. Once the RNA was shown to be DNA free, it was either stored at -80°C or immediately used for reverse transcriptase quantitative PCR (RT-qPCR) (see below).

Reverse transcription and RT-qPCR. Superscript III RT from Invitrogen (Carlsbad, CA) was used to reverse transcribe 1 μ g of DNA-free RNA for each sample analyzed. The reaction mixture contained 1 μ g of RNA, 200 ng random primer hexamers, and buffer supplied by Invitrogen in a final reaction volume of 20 μ l. The RT reaction was performed in a PTC thermocycler (MJ Research Inc., St. Paul, MN). The RNA was denatured at 65°C for 5 min, the reaction mixture assembled, and the reaction allowed to proceed for 44 min at 55°C before being terminated by a 70°C incubation for 15 min (to inactivate the RT). The single-stranded cDNA product of the reaction was diluted 1:10 in nuclease-free water

(final volume, 200 μ l), and 2 μ l was used in a 20- μ l qPCR reaction, as previously described (19). The specificity of the qPCR was evaluated by performing a temperature melt curve analysis; a single sharp peak on the melt curve indicates the synthesis of a single specific product. The relative level of each specific transcript was quantified by comparing the cycle threshold (C_T) values, determined for each PCR, with a standard curve of C_T values generated using known amounts of DNA for the same target gene, over a 10-fold dilution series (42). Specific primers, shown in Table 1, were designed to amplify ~200-bp segments of DNA from *phoA*, *phoD*, *phoX*, *pstS-1*, *pstS-2*, *phnC-1*, *phnC-2*, *phnD-1*, *phnI*, and *phnJ*. At least two biological and three technical replicates were performed for all RT-qPCRs. The results shown represent the three technical replicates (with the standard deviations) for one of the biological replicates. However, the trends and values for the qPCRs were very similar for the second biological replicate.

Growth and physiology of MePhn-acclimated cultures. Growth kinetics and P starvation responses of OS-B' cells acclimated to MePhn were monitored and compared to responses of nonacclimated cells. The acclimated cells were defined as those grown on MePhn as the sole P source for >3 weeks, while nonacclimated cells were maintained on +P medium. Both MePhn-acclimated and nonacclimated cells were grown to late logarithmic phase, washed with P-free medium, and then used to start new cultures at a low cell density (10⁶ cells/ml, or an OD₇₅₀ of 0.020 to 0.025); the cells were subcultured into +P, -P, -P+MePhn, and +P+MePhn media (biological triplicates were used). APase activities and whole-cell absorbance spectra were routinely monitored during cell growth. The loss of acclimation to MePhn was evaluated at various times following the replacement of MePhn with P_i in cell cultures that had been growing for at least 1 month on MePhn as sole P source.

RESULTS

Putative Pho regulon genes. The two completely sequenced genomes of the *Synechococcus* sp. isolated from the hot spring microbial mats of Yellowstone National Park were examined for putative *pho* genes. Based on the annotation available through GenBank (analysis updated June 2008) and prior knowledge of well-studied Pho regulon genes, we identified 42 genes in the OS-B' genome (23 of which have homologs in OS-A) that are putative members of the Pho regulon (Table 2). Recently, an in silico analysis of Pho regulons in cyanobacteria was performed by Su et al., who used a scanning algorithm designed to search for putative Pho box sequences (sequences that bind PhoB) based on those that had been characterized experimentally in various bacteria and cyanobacteria (44). These computational predictions of PhoB binding sites were also used for *pho* gene identifications in the OS-A and OS-B' genomes. According to this analysis, the predicted cyanobacterial Pho box sequences are comprised of three tandem direct repeats of 8 bp with the consensus sequence CTTAACCT and

TABLE 2. Putative Pho regulon genes of the OS-B' genome^a

Name of gene(s)	Locus tag	Putative encoded protein	Closest homolog	% AAID
<i>phoR</i>	CYB_0858	Sensor histidine kinase	<i>Nostoc</i> strain PCC 7120	41
<i>phoB</i>	CYB_2856	Response regulator	<i>Gloeobacter violaceus</i> PCC 7421	75
<i>phoA</i>	CYB_1198	Alkaline phosphatase	<i>Chlorobium chlorochromatii</i> CaD3	45
<i>phoX</i>	CYB_1988	Alkaline phosphatase	<i>Hahella chejuensis</i> KCTC 2396	54
<i>phoD</i>	CYB_0684	Phosphodiesterase	<i>Bacillus subtilis</i>	28
<i>surE-1</i>	CYB_0884	Acid phosphatase	<i>Thermosynechococcus elongatus</i> BP-1	60
<i>surE-2</i>	CYB_1427	Acid phosphatase	<i>Lyngbya</i> strain PCC 8106	51
<i>phoH</i>	CYB_2320	PhoH family protein	<i>Thermosynechococcus elongatus</i> BP-1	61
<i>npp</i>	CYB_0274	5'-Nucleotidase phosphatase	<i>Cyanothece</i> strain PCC 7424	53
<i>nucH</i>	CYB_2765	Putative secreted nuclease	<i>Roseiflexus castenholzii</i> DSM 13941	46
<i>ppx</i>	CYB_1493	Exopolyphosphatase	<i>Anabaena variabilis</i> ATCC 29413	58
<i>ppk</i>	CYB_2082	Polyphosphate kinase	<i>Cyanothece</i> strain PCC 8801	62
<i>pstS-1, pstC-1, pstA-1, pstB-1</i>	CYB_1077-74	High-affinity ABC-type Pi transporter	<i>Cyanothece</i> strain CCY 0110 ^b	50, 51, 53, 70
<i>pstS-2, pstC-2, pstA-2, pstB-2</i>	CYB_1915-12	High-affinity ABC-type Pi transporter	<i>Cyanothece</i> strain ATCC 51142 ^b	45, 50, 70, 70
<i>phoU</i>	CYB_2526	Regulatory protein	<i>Nostoc</i> strain PCC 7120	61
<i>phnC-1, phnD-1, phnE-1</i>	CYB_0159-61	Phn ABC-type transporter proteins	<i>Cyanothece</i> strain PCC 8801 ^b	44, 31, 45
<i>phnG-phnM</i>	CYB_0162-68	C-P lyase	<i>Roseiflexus</i> strain RS-1 ^b	48, 32, 38, 59, 45, 41, 39
<i>phnD</i> ; ^c <i>phnD-2, phnD-3, phnC-2, phnE-2, phnE-3</i>	CYB_1464-69	Phn ABC-type transporter proteins	<i>Dinoroseobacter shibae</i> DFL 12 ^b	69, 51, 53, 72, 54, 56
<i>phnE-4, phnD-4, phnC-3</i>	CYB_0012-11, 09	Phn ABC-type transporter proteins	<i>Cyanothece</i> strain PCC 7424 ^b	36, 27, 69
<i>ugpB, ugpA</i>	CYB_2477-78	Glycerol-3-phosphate transporter	<i>Deinococcus geothermalis</i> DMS 11300 ^b	39, 46

^a Gene names, locus tags, predicted products, and percent AAID to the closest bacterial homolog in all available sequenced organisms (excluding OS-A) are given. Genes associated with putative operons are grouped in the list. Genes shown in bold are preceded by a high-ranking predicted Pho box (44).

^b For gene clusters, we show the percent AAID relative to the respective homologs from a single organism.

^c PhnD had the highest AAID to the *Sinorhizobium meliloti* 1021 homolog.

which are separated by A/T-rich 3-bp linker sequences. This differs from the well-defined Pho box of *E. coli*, which consists of at least two tandem direct repeats (Pho boxes) of 8 bp with the consensus sequence CTGTACTA separated by an A/T-rich 3-bp linker that is located 10 bp upstream of the $-\sigma^{70}$ TAN₃T/A binding site (49).

The 42 putative *pho* genes identified on the OS-B' genome were examined for the presence of a putative PhoB binding site, based on the consensus sequence (44) (Fig. 1A, inset). Six genes have highly ranked Pho box sequences upstream of their coding region, and the genes downstream of them are likely to be part of a Pho-regulated operon (Table 2). Based on the in silico Pho box predictions, it is likely that there are several other genes, not investigated in this study, that may be part of the Pho regulon (44). The OS-A and OS-B' orthologs associated with the Pho regulon, based on a mutual best BLAST hit analysis (updated June 2008), share high identity at the nucleotide (NAID) (83 to 98%) and amino acid (AAID) (86 to 98%) levels (see Table S1 in the supplemental material). There is also a relatively high conservation of predicted Pho box sequences between orthologs of OS-A and OS-B' (40; Z. Su, personal communication). This is not surprising, since these two isolates are closely related (3). On the other hand, we found that there is relatively low identity between the OS-A and OS-B' Pho regulon genes and their closest putative orthologs in other cyanobacteria or bacteria (from 27 to 75% AID), as shown in Table 2.

Two-component regulatory system. The OS-A and OS-B' genomes encode the sensor histidine kinase PhoR (CYA_2352

and CYB_0858) and the response regulator PhoB (CYA_1033 and CYB_2856), which constitutes the two-component system that controls the activity of the P_i starvation-responsive Pho regulon. There is relatively low identity of the putative PhoB/PhoR polypeptides of OS-B' to the analogous proteins of *E. coli*; the highest identity is to PhoB of *Gloeobacter violaceus* PCC 7421 (75% AAID) and PhoR of *Nostoc* strain PCC 7120 (41% AAID) (Table 2).

High-affinity ABC-type P_i transport systems. The OS-A and OS-B' genomes both contain two copies of a putative *pstSCAB* operon that are located at different genomic locations: *pstS-1, pstC-1, pstA-1, and pstB-1* (CYA_1558-CYA_1555 and CYB_1077 to CYB_1074) and *pstS-2, pstC-2, pstA-2, and pstB-2* (CYA_1735 to CYA_1732 and CYB_1915 to CYB_1912) (Table 2). Moreover, the putative PhoB binding site upstream of the second *pstS-2 C-2 A-2 B-2* gene cluster in OS-B' (CYB_1915 to CYB_1912) was the highest-ranked Pho box sequence predicted by in silico analysis (44). The regulatory gene *phoU* (CYA_0182 and CYB_2562) is not associated with either of the *pstSCAB* operons (in either OS-B' or OS-A).

Phosphatases. There are at least seven putative phosphatases (*phoA, phoX, phoD, surE-1, surE-2, nucH, and npp*) encoded by the OS-A and OS-B' genomes (56). High-scoring Pho box sequences were predicted upstream of the *phoA, phoX, phoD, and nucH* genes in OS-B', suggesting that they are likely to be regulated by PhoB binding (44). The *phoA* gene (CYA_0781 and CYB_1198), present in both OS-A and OS-B', encodes an APase with a predicted molecular mass of 48 kDa. The highest identity/similarity of the OS-B' PhoA is to

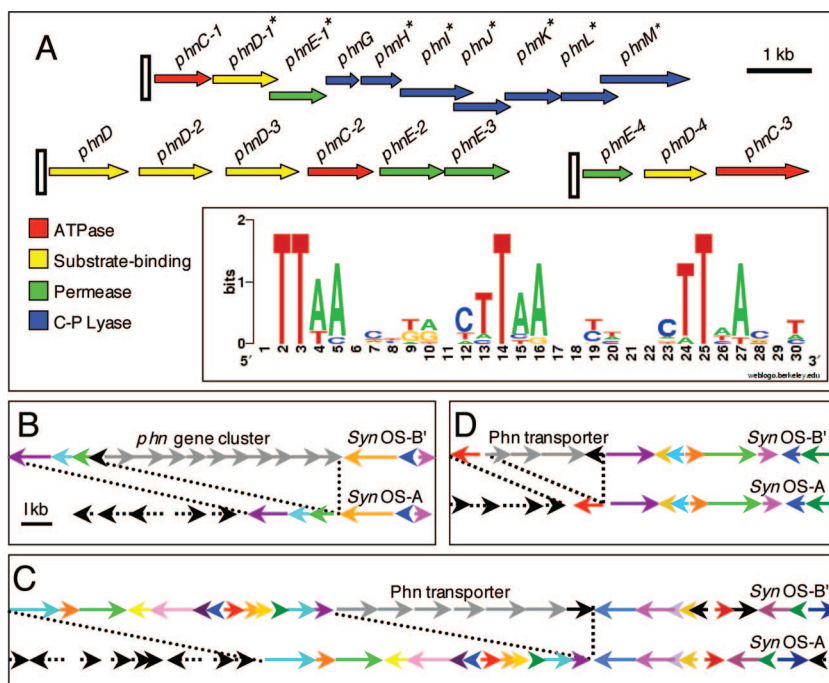


FIG. 1. Genomic organization of *phn* genes and comparison of the regions flanking the *phn* gene clusters in OS-B' and OS-A. (A) The top row of genes shows the main *phn* gene cluster of OS-B', with the ABC phosphonate (Phn) transporter (*phn C-1*, *phn D-1*, and *phn E-1*) and the C-P lyase (*phnG*-*phnM*) genes. The bottom row shows the second (*phnD*, *phn D-2*, *phn D-3*, *phn C-2*, and *phn E-3*) and third (*phnE-4*, *phnD-4*, and *phnC-3*) *phn* clusters. See Table 2 for accession numbers. The Phn transporter genes include the ATPase component (*phnC*; red), the substrate binding protein (*phnD*; yellow), and the membrane permease component (*phnE*; green). A putative Pho box is located upstream of *phnC-1*, *phnD*, and *phnC*. Asterisks indicate genes that overlap with the next contiguous gene. (Inset) Logo representation of the profile of the top 10 ranked Pho boxes of OS-B', as predicted by phylogenetic footprinting (40). The logo was generated by using the Weblogo server (<http://weblogo.berkeley.edu/logo.cgi>). (B) Organization of flanking regions around the main *phn* gene cluster (gray arrows) in OS-B' compared to the analogous region in OS-A. (C) Organization of flanking regions around the second *phn* cluster (*phnD*, *phnD-2*, *phnD-3*, *phnC-2*, *phnE-2*, and *phnE-3*) in OS-B' compared to the analogous region in OS-A. (D) Organization of flanking regions around the third *phn* cluster (*phnE-4*, *phnD-4*, and *phnC-3*) in OS-B' compared to the analogous region in OS-A. Gray arrows represent the *phn* genes, and solid colored arrows (but not black arrows) indicate syntenic sequences in OS-A relative to OS-B', while the broken arrows indicate genes where there is a break in synteny between the two genomes. Black arrows in the OS-B' genome represent genes not present in OS-A. The scale is indicated in panel B.

the APase of the symbiotic green sulfur bacterium *Chlorobium chlorochromatii* CaD3 (45% AAID, 65% amino acid similarity). Both the OS-A and OS-B' genomes contain a putative *nucH* homolog (CYA_0117 and CYB_2765), although it is not contiguous with *phoA*, and the OS-B' NucH is most similar to the putative secreted nuclease of the thermophilic, filamentous, anoxygenic phototroph *Roseiflexus castenholzii* DSM 13941 (46% AAID), which is also present in the OS microbial mat.

The OS-A and OS-B' genomes also encode a putative extracellular alkaline phosphatase, PhoX (CYA_1696 and CYB_1988), which is most similar to the PhoX of the marine, gamma-proteobacterium *Hahella chejuensis* KCTC 2396 at 54% AAID. The *phoX* gene is not present in all completely sequenced cyanobacterial genomes; it has been identified on the genomes of *Gloeobacter violaceus*, *Nostoc* strain PCC 7120, and *Synechococcus* strain WH 8102. Genes encoding the acid phosphatases SurE-1 (CYA_0967 and CYB_0884) and SurE-2 (CYA_0017 and CYB_1427) are also present on the OS-A and OS-B' genomes, and the orthologs exhibit 95% and 92% AAID to each other, respectively (see Table S1 in the supplemental material). These phosphatases are thought to function in liberating P_i from phosphate esters when cells enter stationary phase (38).

Poly(P) synthesis and utilization. The OS-A and OS-B' genomes contain the *ppk* gene (CYA_2477 and CYB_2082), which encodes polyphosphate kinase 1, and the *ppx* gene (CYA_2432 and CYB_1493), which encodes an exopolyphosphatase (Table 2). The presence of the *ppk* and *ppx* genes suggests that these hot spring cyanobacteria can acclimate to environmental P fluctuations by modulating the formation and metabolism of cellular poly(P) reserves. The putative PPK and PPX that are encoded in the OS-A and OS-B' genomes are 94% and 91% identical to each other, respectively, but the OS-B' homolog has only 62% AAID to the putative PPK of *Cyanothece* strain PCC 8801 and 58% AAID to the PPX of the filamentous, heterocyst-forming cyanobacterium *Anabaena variabilis* ATCC 29413.

The *phn* gene clusters. The OS-B' genome contains a cluster of contiguous genes associated with Phn uptake and metabolism. This cluster, designated the main *phn* cluster, represents a putative operon consisting of 10 genes (*phnC-1*, *phnD-1*, *phnE-1*, *phnG*, *phnH*, *phnI*, *phnJ*, *phnK*, *phnL*, and *phnM*; CYB_0159 to CYB_0168) and spans ~8 kb. The *phn* genes of OS-B' are organized similarly to that of the well-characterized 14-gene *phn* operon of *E. coli*, with genes encoding the transport proteins at the 5' end of the putative operon followed by

genes encoding the C-P lyase (Fig. 1A, top gene cluster) (27). However, these polypeptides associated with Phn utilization in OS-B' show relatively low AAID (between 31 and 59%) to polypeptides encoded by other *phn* genes and are not present in the OS-A genome (Table 2). Neither the OS-B' nor the OS-A genome contains putative homologs of other genes that would allow for Pho-independent phosphonate catabolism, including *phnA*, *phnB*, *phnW*, *phnX*, and *phnY*.

Interestingly, 8 of the 10 genes in the OS-B' *phn* operon overlap by 7 to 31 bp, with overlapping sequences found between *phnD-1* and *phnE-1* (31 bp), *phnH* and *phnI* (15 bp), *phnI* and *phnJ* (16 bp), *phnJ* and *phnK* (7 bp), and *phnL* and *phnM* (19 bp). Putative Pho box elements have been predicted upstream of the *phn* gene cluster; the sequence of the PhoB binding site upstream of this cluster was ranked the second highest relative to all such sites on the OS-B' genome (44).

In addition to the main *phn* gene cluster, there are two additional clusters ("secondary" clusters) with genes encoding putative Phn transporter components (Fig. 1A, gene clusters in the second row). The second *phn* cluster contains six transport-related genes (*phnD*, *phnD-2*, *phnD-3*, *phnC-2*, *phnE-2*, and *phnE-3*; CYB_1464 to CYB_1469), while the third *phn* cluster contains three genes (*phnE-4*, *phnD-4*, and *phnC-3*; CYB_0012 and -11 and CYB_0009). There is no C-P lyase gene associated with either of these clusters. None of these *phn* genes is present in the OS-A genome, and the OS-B' Phn polypeptides show relatively low AAID to polypeptides encoded by *phn* genes in other organisms (Table 2). Putative Pho box sequences were also predicted upstream of these *phn* clusters, albeit with much lower scores, based on a scanning algorithm (Z. Su, personal communication), than that obtained for the Pho box sequence of the main *phn* gene cluster. The putative Phn transporter proteins encoded by the secondary clusters exhibit low AAID to potential paralogs of the main cluster (see Table S2 in supplemental material). However, the polypeptides encoded by the secondary clusters have higher AAIDs to Phn transport polypeptides than to any of the other transport polypeptides, including those associated with P_i transport in various bacteria (data not shown). These results suggest that the secondary cluster polypeptides are involved in Phn uptake, although they may be tailored for the uptake of specific Phn substrates.

While comparison of the two sequenced genomes of the *Synechococcus* isolates from OS indicates that the OS-A genome lacks any gene or gene fragment with significant similarity to *phn* genes, the genomic region that flanks the 8-kb main *phn* gene cluster of OS-B' is present in OS-A; the genes that immediately border the *phn* genes are contiguous in the OS-A genome (Fig. 1B). Similarly, the regions flanking the second (Fig. 1C) and third (Fig. 1D) *phn* clusters in OS-B' are also contiguous in the OS-A genome. The proteins encoded by these flanking genes are relatively highly conserved between the genomes (ranging from 78 to 96% AAID), which may indicate mobility of *phn* gene clusters within the mat microbial population and explain the presence of these genes in one isolate and their absence in the other. However, it is difficult to determine how these clusters may have been transferred; a single transposase-like gene (CYB_0018) is found on the genome contiguous to one of the putative Phn transporter clusters (CYB_0009, CYB_0012-11).

P deprivation alters cell growth and physiology. The abilities of axenic OS-B' cultures to cope with P deprivation and to utilize MePhn as a sole P source were analyzed. OS-B' grown in +P medium had a doubling time of ~24 h up to the point at which the cells reached stationary phase, going from $\sim 1 \times 10^6$ to nearly 1×10^8 cell/ml (between six and seven doublings) in 192 h (Fig. 2A). Cells in +P medium showed low-level, constitutive APase activity ($<0.02 \mu\text{g pNPP hydrolyzed/h/}1 \times 10^6$ cells), which did not change significantly throughout the growth period (Fig. 2B). When the +P cells were transferred to -P conditions, growth stopped after ~72 h (i.e., ~three to four doublings) at a density of $\sim 1 \times 10^7$ cells/ml, as shown in Fig. 2A, while the APase activity reached maximal levels ($\sim 0.3 \mu\text{g pNPP hydrolyzed/h/}1 \times 10^6$ cells) at ~48 h following the transfer to -P conditions; this level of activity was sustained throughout the stationary phase of growth (Fig. 2B). Furthermore, P starvation of OS-B' caused changes in whole-cell absorption spectra that have been associated with macronutrient deprivation in other cyanobacteria (8); there was a decrease in both chl *a* (maximal absorbance at 430 and ~675 nm) and PBP pigments (maximal absorbance at 620 nm) on a per cell basis, measured after 96 h in -P medium (Fig. 2C). This loss of photosynthetic pigment is most likely due to dilution of PBP and chl after termination of synthesis, assuming that P_i starvation affects the photosynthetic apparatus of OS-B' in a manner similar to that of *Synechococcus* strain PCC 7942 (8). Further quantifications of pigments and their synthesis and turnover would be required to clearly address this point.

When cells were transferred to -P medium supplemented with MePhn as the sole P source, they ceased growth within 24 h; this is in contrast to growth of OS-B' in medium lacking P, in which cells doubled three to four times (Fig. 2A). These cultures also exhibited markedly lower APase activity relative to the -P cultures (Fig. 2B). When both MePhn and P_i were included in the medium, cell division continued for at least 192 h. The growth rate for the +P+MePhn cells was comparable to that of the +P cells for the first 24 h and then slowed relative to cells grown in +P medium between 24 and 120 h. Approximately the same final cell densities for the +P+MePhn and the +P cells were attained when the experiment was terminated at 192 h. Both the -P and the -P+MePhn cultures stopped growing sooner than either the +P or +P+MePhn cultures and never reached as high a cell density. The lowest cell density was observed for the -P+MePhn-grown cells, which appeared to enter stationary phase at a density of 2.5×10^6 to 3.2×10^6 cells/ml. Like the -P cultures, the -P+MePhn cultures exhibited a decrease in chl *a* and PBP content, based on whole-cell absorbance spectra. However, only the -P+MePhn cultures showed a notable change in absorbance at 480 to 550 nm, suggesting an increase in carotenoid accumulation after 96 h of starvation (Fig. 2C). We conclude that MePhn is inhibitory to cell growth over the time period of 200 h and exacerbates the physiological responses of cells to the starvation conditions.

Pho regulon transcripts. We used qPCR with specific primers (Table 1) to quantify the level of transcripts for several genes of OS-B' of the putative Pho regulon. The cultures were initially grown under nutrient-replete conditions and subsequently transferred to four different media, as done for the experiment presented in Fig. 2: +P (nutrient replete medium), -P (no P added to medium), +P+MePhn (nutrient replete

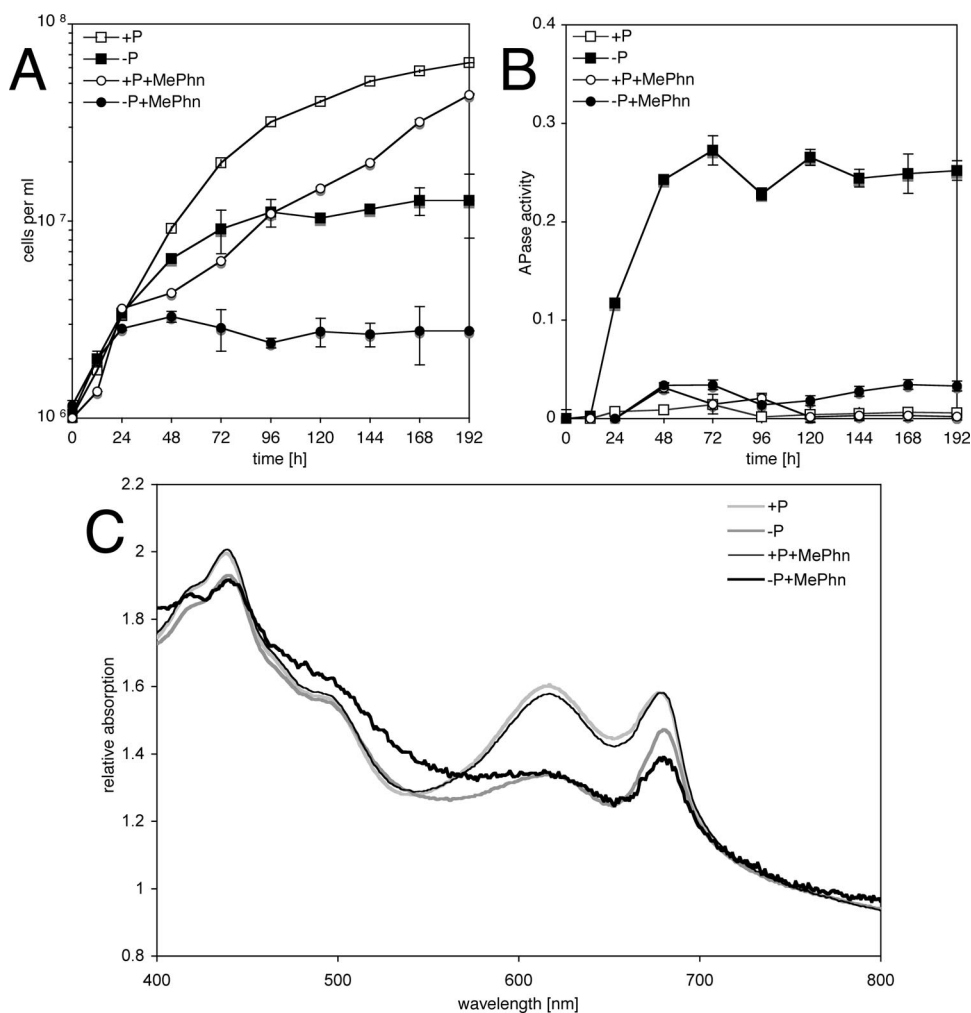


FIG. 2. Growth response, APase activity, and absorbance spectra of OS-B' under various P conditions. (A) Late-logarithmic-phase cells grown in +P medium were transferred growth under four different conditions: (i) +P, (ii) -P, (iii) +P medium with 0.5 mM MePhn (+P+MePhn), and (iv) -P medium with 0.5 mM MePhn (-P+MePhn). Growth of all cultures was monitored for ~192 h. Note the log scale on the y axis for cells per ml data. (B) APase activity and growth measurements were quantified in cell cultures once every 24 h. The APase activity was measured as μg of pNPP hydrolyzed per h per 1×10^6 cells. (C) Whole-cell absorbance spectra of each culture between the wavelengths of 400 and 800 nm, normalized to the OD_{750} at 96 h after cell transfer. Results for growth and APase activity show the means and standard deviations (error bars) from measurements taken from biological triplicates.

supplemented with MePhn), and -P+MePhn (MePhn as the sole P source). The density of the cells transferred to the new growth conditions was very low ($<10^6$ cells/ml) to ensure that the cells were in the logarithmic phase of growth and that they would have the capacity to maintain logarithmic growth over the course of the experiment.

The ratios of individual transcripts in cells grown under the different P conditions tested are shown in Fig. 3 and 4. A similar trend in transcript accumulation was obtained for all putative Pho regulon transcripts that were quantified. This trend was also verified by a biological replicate (data not shown), and all technical replicates had standard deviations from the mean of 5% or less. An increase in transcript levels was observed after 24 h under -P conditions (expressed as the ratio of -P/+P or -P+MePhn/+P+MePhn transcript levels) and remained high at 72 h of P deprivation. The increase in transcript abundance observed in cells transferred to -P+MePhn relative to +P+MePhn was gen-

erally not as great as that observed for cells transferred to -P relative to +P, particularly at the 72-h time point. As shown in Fig. 3 (top panel), transcripts from *phoA* (CYB_1198) and *phoD* (CYB_0684) were both sixfold higher after 24 h of P starvation. The highest transcript levels were observed 72 h after the transfer of cells to -P conditions, with 16-fold and 21-fold changes for *phoA* and *phoD* transcripts, respectively. Transcripts for the *phoX* alkaline phosphatase (CYB_1988) increased within 24 h and continued to accumulate over the time course in -P relative to +P conditions; the increase in relative transcript level was >200 -fold after 72 h.

Similar trends in transcript abundance were observed for the Pho regulon genes encoding the periplasmic P_i binding proteins associated with the high-affinity transport systems *pstS-1* (CYB_1074) and *pstS-2* (CYB_1911) (Fig. 3, middle panel), although the increase for *pstS-2* was significantly more than for *pstS-1*. Transcripts for *phnC-2* (CYB_1467), encoding the ATP

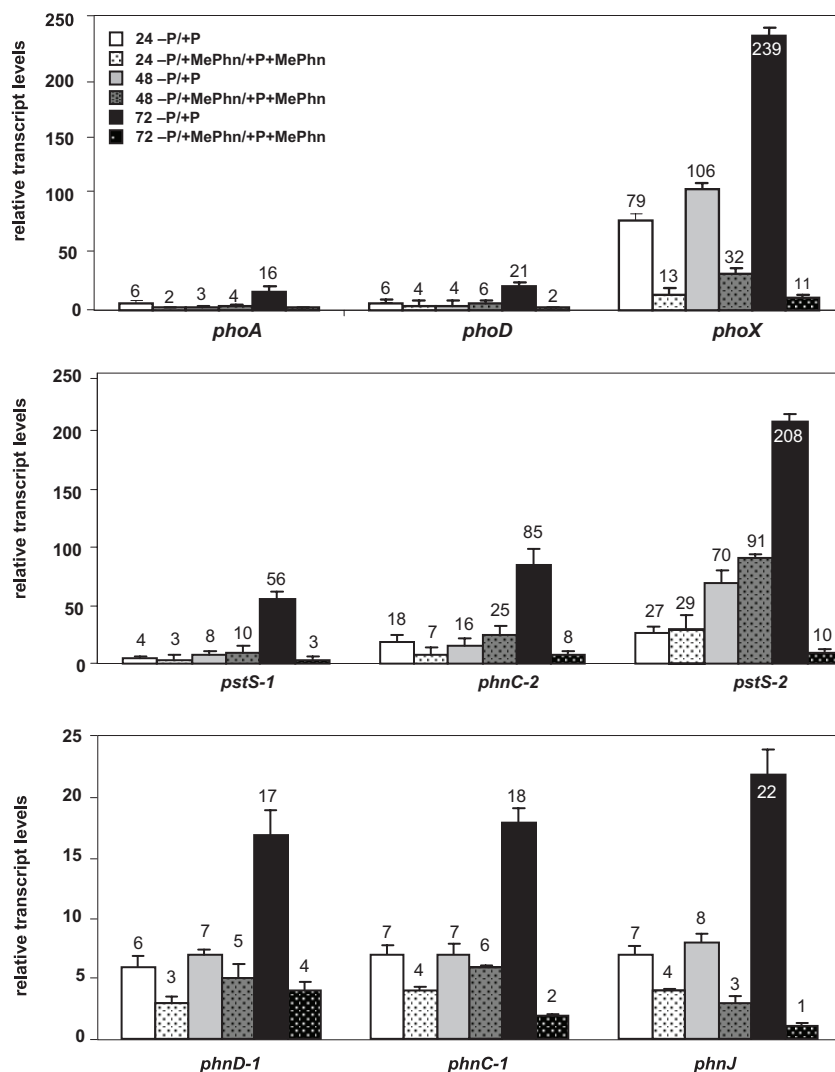


FIG. 3. Quantification of relative Pho regulon transcript accumulation following P_i starvation. Specific Pho regulon transcripts, including those of the *phn* gene cluster, were measured over 24-h intervals under various P conditions. The top panel represents transcripts for genes encoding phosphatases, the middle panel is transcripts for genes associated with transport systems, and the bottom panel is transcripts for genes involved in Phn transport and metabolism. For each gene, relative transcript levels, presented as a ratio under $-P$ and $+P$ conditions, were determined at 24, 48, and 72 h following the transfer of cells to the new growth medium. In the presence of MePhn, relative transcript levels representing $-P+MePhn$ compared to $+P+MePhn$ are shown at 24 h, 48 h, and 72 h. The qPCR results show the means and standard deviations (error bars) for data from three technical replicates.

binding component of the Phn transporter, also exhibited a >80 -fold increase by 72 h of P deprivation.

In a separate experiment, cells were grown in $-P$ medium for 72 h and then were either supplemented with P_i (an addition to 0.77 mM P_i) or maintained in $-P$ medium; growth was continued for an additional 24 h for each of the cultures before transcript analysis. The addition of P_i to the cultures after 72 h of P starvation caused a rapid decrease in putative Pho regulon transcript levels within 24 h; decreases were observed for the phosphatase (*phoA*, *phoD*, and *phoX*) and ABC-type transporter periplasmic substrate-binding protein transcripts (*pstS-1*, *phnC-1*, and *phnC-2*) (Fig. 4) in comparison to the respective transcript levels at 72 h (Fig. 4). As expected, the transcript levels remained high for cells maintained under $-P$ conditions for the additional 24 h (Fig. 4), although in some

cases there was some decrease. This decrease could reflect a reduction in the growth and metabolism at the later time following the imposition of P deprivation.

The *phn* gene cluster is part of the Pho regulon. We also monitored changes in several transcripts from the *phn* gene clusters during growth in $+P$ and $-P$ growth medium, as well as in the presence and absence of MePhn (Fig. 3, middle and bottom panels). After 72 h in $-P$ medium, there was an increase in transcript levels of 18-fold for *phnC-1* and 17-fold for *phnD-1* (Fig. 3, bottom panel); these genes are likely part of the same operon (Fig. 1A, first row). Transcripts of the second Phn transport cluster exhibited a greater change in relative transcript levels than those of the main *phn* cluster; there was a >80 -fold increase of *phnC-2* by 72 h (Fig. 3, middle panel). An increase in transcripts from genes encoding the C-P lyase

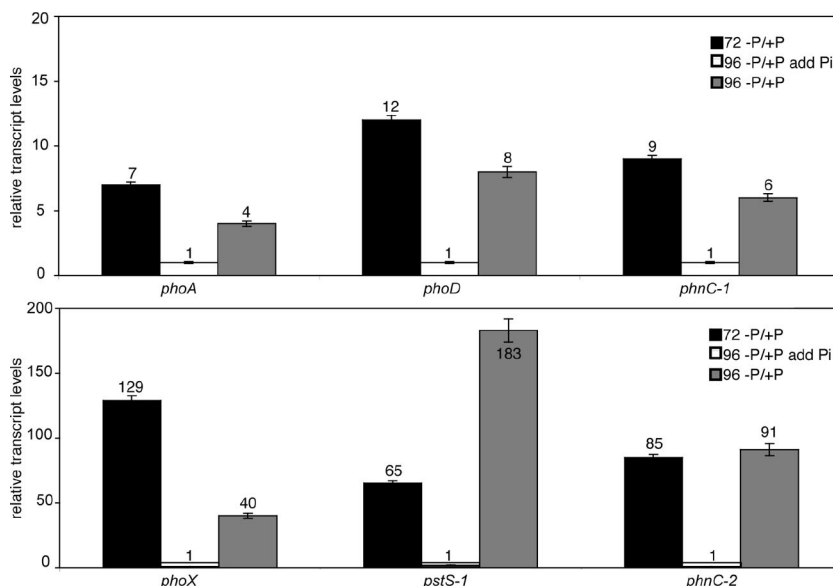


FIG. 4. Quantification of Pho regulon transcript accumulation after P_i addition to P-starved cultures. P_i was added back to the growth medium after 72 h of starvation, and transcript levels were measured (as described in Fig. 3) at 96 h (i.e., 24 h after adding P_i back). Relative transcript levels are shown that compare $-P$ to $+P$ after P_i addition and the control, i.e., $-P$ compared to $+P$ prior to P_i addition but with no P_i added back. The qPCR results show the means and standard deviations (error bars) for data from three technical replicates.

was also observed when the cells experienced P deprivation. Transcripts for *phnJ* increased 22-fold after 72 h of growth in $-P$ compared to $+P$ medium. Similarly, an increase of transcript levels was observed for cells grown in $-P+MePhn$ relative to cells grown in $+P+MePhn$, although the ratio was generally lower than that observed for cultures that were not supplemented with MePhn, and the 72-h time point generally exhibited the lowest ratio (Fig. 3, middle and bottom panels). Finally, the addition of P_i to the P-starved cultures caused a rapid decrease in *phn* gene transcript accumulation within 24 h, as quantified at the 96-h time point for *phnC-1* and *phnC-2* (Fig. 4); the transcripts remained high in cultures to which no P_i was added (Fig. 4).

MePhn addition alters Pho regulon transcript levels. To examine how MePhn affects levels of transcripts after cells are transferred to $-P$ medium, we compared levels of transcripts for the putative Pho regulon genes following 72 h of growth in $-P$ medium relative to $-P+MePhn$ medium; the ratios of the

transcript abundances measured under these two conditions are presented in Table 3. For all investigated genes that were affected by P starvation, the transcript levels following 72 h of growth on $-P$ medium were between 4- and 18-fold higher than in cells grown for 72 h in $-P$ medium supplemented with MePhn. These results demonstrate that MePhn suppresses accumulation of Pho regulon transcripts during P deprivation, although as demonstrated in Fig. 3, there is still an increase in transcript levels when cultures supplemented with MePhn are placed into medium lacking P.

Acclimation to MePhn and its utilization. The capacity of OS-B' to metabolize MePhn as a sole P source was tested. We observed that cells transferred to $-P+MePhn$ showed a slight initial growth during the first 24 h and then stopped growing, with no additional growth for the first 16 to 20 days (Fig. 2A). The cells also showed significant bleaching (Fig. 2C), indicating a loss of photosynthetic pigments on a per cell basis. However, after ~20 days in $-P+MePhn$ medium, the OS-B' cells slowly appeared to regain pigmentation and grow. During this 3- to 4-week "acclimation" period, the cells attained doubling times comparable to those of cells kept in nutrient-replete medium. After the $-P$ cells supplemented with MePhn reached stationary phase, we considered them acclimated to growth on MePhn and they were subcultured into fresh $-P$ medium supplemented with MePhn.

To further examine this phenomenon, we initiated growth experiments in which OS-B' cultures were either acclimated to phosphonate (maintained in $-P+MePhn$ medium) or maintained under nonacclimated conditions (in $+P$ medium with no exposure to MePhn). Both the nonacclimated and acclimated cells were subcultured during logarithmic growth into $+P$, $-P$, or $-P+MePhn$ medium, to investigate the effects of MePhn on the growth of both nonacclimated and acclimated cells.

TABLE 3. Effect of MePhn on transcript accumulation^a

Gene	Relative transcript level (at 72 h)
<i>phoX</i>	18
<i>phnJ</i>	14
<i>pstS-2</i>	11
<i>phnC-1</i>	8
<i>phoA</i>	8
<i>pstS-1</i>	7
<i>phnC-2</i>	5
<i>phnD-1</i>	5
<i>phoD</i>	4

^a Comparison of relative transcript levels from cells grown under $-P$ conditions relative to $-P+MePhn$ conditions after 72 h of starvation. Transcripts were quantified for all investigated genes of the OS-B' Pho regulon, as illustrated in Fig. 3 and 4.

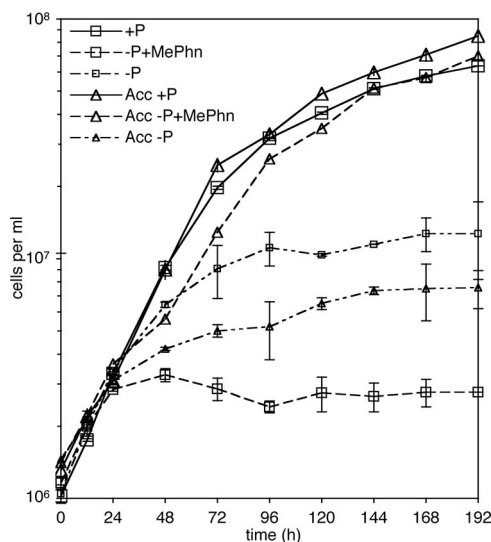


FIG. 5. Growth of cultures in MePhn. Growth responses of MePhn-acclimated (triangles) and nonacclimated (squares) OS-B' cells to +P, -P, and -P+MePhn conditions. The graph shows the means and standard deviations (error bars) from measurements taken from biological triplicates. Starting cultures were initially grown to logarithmic phase in either +P or -P+MePhn medium for 20 days. Note the log scale on the y axis for cells per ml data.

Placing nonacclimated OS-B' cells into -P+MePhn medium strongly inhibited cell division compared to cells placed in -P medium (Fig. 2A and 5). Nonacclimated cells stopped growing at 24 to 48 h after their transfer to -P+MePhn medium. In contrast, cultures transferred to -P medium (not supplemented with MePhn) kept growing for ~72 h and reached much higher cell densities (Fig. 2A and 5). Growth characteristics of the Phn-acclimated cells were also monitored in +P, -P, and -P+MePhn media (Fig. 5). The Phn-acclimated OS-B' cultures exhibited a doubling time of ~24 h in the presence of +P or -P+MePhn; the doubling time of the acclimated cells in -P+MePhn was only slightly lower than that of acclimated cells grown in +P medium. Phn-acclimated cells continued to double with P_i or MePhn supplied as a P source and reached stationary phase at ~120 h. The growth kinetics of the Phn-acclimated cells in +P or -P+MePhn was similar to that of nonacclimated cells grown in +P medium. Furthermore, the Phn-acclimated cells grown in -P+MePhn medium continued to divide over the entire 192-h growth period and reached a final cell density comparable to that of either nonacclimated or acclimated cells grown in +P medium. These results suggest that the acclimated cells can use Phn as a sole P source and, furthermore, that utilization of this P source results in rates of growth and final cell densities that are comparable to those observed when the cells are grown in medium containing P_i .

DISCUSSION

Studies of the Pho regulon and P starvation responses in model bacterial systems, including *E. coli* (46, 49) and *Synechocystis* sp. strain PCC 6803 (13, 45), have established a wealth of information that has guided us in investigations of P starvation

responses in the thermophilic cyanobacterium *Synechococcus* OS-B' isolates. Complete genome sequence information coupled with physiological studies and transcript analyses of OS-B' have yielded insights as to how thermophilic cyanobacteria cope with P limitation and may utilize alternative P sources within the microbial mat consortium. This investigation demonstrated that genes of the putative Pho regulon of OS-B', a key photosynthetic organism in the microbial mats of Mushroom Spring and Octopus Spring, are responsive to P starvation. The genome also contains *phn* gene clusters, which appear to be part of the Pho regulon and are likely involved in the ability of the cells to grow on MePhn (or other Phns in the environment) when no other P source is available. Indeed, one of the major findings of this study is that OS-B' is capable of acclimating to P starvation conditions by developing the ability to utilize MePhn as a sole P source.

Initially, we addressed the question of whether OS-B' is physiologically adapted to cope with P limitation. Axenic cultures of OS-B' continued to progress through three to four cell divisions after P_i was removed from the growth medium; this contrasts with the results obtained for other microorganisms, such as *E. coli*, which rapidly enter stationary phase upon removal of P from the medium (35). The continued growth of OS-B' after removal of P from the medium suggests that the storage and metabolism of intracellular poly(P) may be one mechanism that enables the organism to cope with low exogenous P_i , as has been demonstrated for other cyanobacteria (12, 21, 22). Consistent with this possibility, we have measured poly(P) levels and found that there are large poly(P) pools present in OS-B', but these levels are much lower in cells maintained on MePhn (M. R. Gomez-Garcia, M. Adams, A. Grossman, and D. Bhaya, unpublished data). A similar result has also recently been reported for the chemolithoautotroph *Acidithiobacillus ferrooxidans*, which is also able to use MePhn as an alternative P source; during growth on Phn there is a drastic reduction in poly(P) levels relative to the level in cells grown in the presence of P_i (48).

P_i limitation of OS-B' was found to elicit the accumulation of extracellular APase activity and increased levels of transcripts encoding putative phosphatases. The phosphatase genes that appeared to be activated at the transcriptional level included *phoA*, *phoD*, and *phoX*, which encode enzymes that can cleave P_i from phosphate monoesters and diesters (the transcriptional activities for *nucH*, *surE-1*, *surE-2*, and *npp* were not examined in this study). The PhoX of OS-B' may be responsible for most of the extracellular phosphatase activity assayed during P deprivation, similar to the PhoX of *Pseudomonas fluorescens* Pf0-1, as transcript levels of *phoX* exhibited a much greater change than those of *phoA* or *phoD* following P starvation of OS-B'. The role of PhoX as the primary phosphatase is also in line with the highly ranked Pho box predicted to precede the *phoX* gene (CYB_1198), based on in silico analysis.

Transcripts encoding the high-affinity ABC-type Pst transport systems also accumulated in response to P depletion. Upregulation of both sets of *pstS* transcripts suggests that OS-B' has two P_i transport systems that become more abundant when environmental P_i concentrations decline. In comparison, *E. coli* has a single Pst transport system plus a Pit transport system; the latter is active when P_i is in excess (35).

Examination of 19 complete cyanobacterial genome sequences showed that all contained at least one set of genes encoding the Pst transporter. The genomes of the hot spring *Synechococcus* isolates OS-B' and OS-A, as well as *Anabaena variabilis*, *Synechocystis* strain PCC 6803, *Nostoc* strain PCC 7120, and *Gloeobacter violaceus* PCC 7421, contain two clusters of these genes (44), although it is not clear if both of the Pst transporters encoded on the cyanobacterial genomes have a high affinity for their substrate. None of the genomes of any marine *Prochlorococcus* spp. or *Trichodesmium erythraeum* IMS101 contain two full *pst* transporter gene clusters, although *Prochlorococcus* does contain some duplicate gene homologs. Perhaps selection for a single high-affinity Pst system has occurred in the very stable, low- P_i marine environments, in comparison to other environments where bioavailable P_i may fluctuate more over time and it may be advantageous to have transporters with different kinetic and regulatory characteristics.

Another set of genes involved in P assimilation, which are also responsive to P depletion, are those encoding proteins involved in the transport and assimilation of Phns. Clusters of *phn* genes are present on the genome of OS-B' and, similar to the *phn* operon of *E. coli*, P_i starvation elicits accumulation of *phn* transcripts in OS-B'. Most microorganisms investigated thus far exhibit *phn* gene expression when the cells are starved for P, so there is no apparent requirement for induction of the system by Phn molecules (20). This is also the case for OS-B', for which transcripts of all investigated *phn* genes increase when P is eliminated from the growth medium (no Phn supplementation). However, when MePhn was added to cells deprived of P_i , it caused a depression in the levels of *phn* transcripts, relative to cells exposed to $-P$ conditions without MePhn supplementation. Interestingly, Phn transporters in some bacteria have been shown to have relaxed substrate specificity, with an affinity not only for Phns but also for P_i (54) and other reduced P substrates (55), which raises the possibility that the *phn* genes of OS-B' may encode proteins with the ability to acquire a broader range of substrates than just Phn. This is likely the case for OS-B', which has three separate *phn* clusters on the genome encoding putative Phn transporter components that exhibit low homologies both among themselves and with Phn proteins in other organisms.

While all putative Pho regulon genes of OS-B' are responsive to P_i levels, the polypeptides encoded by these genes in many cases have low AAIDs with the analogous proteins from other bacteria, such as *E. coli*, *Thermosynechococcus elongatus*, and *Roseiflexus* strain RS-1. For example, PhoA of OS-B' is highly divergent from the PhoA in all other bacteria. This contrasts with the acid phosphatases (SurE-1 and SurE-2) of OS-B', which share 60% AAID with the SurE-1 of *Thermosynechococcus elongatus* BP-1 and 51% AAID to the SurE-2 of *Lyngbya* strain PCC 8106. Despite the lack of strong sequence conservation among many bacterial Pho regulon proteins with those of OS-B' and OS-A, there is a relatively high NAID and AAID between the *pho* orthologs of OS-A and OS-B'. Furthermore, many Pho regulon genes that are present in the hot spring cyanobacteria, including *phoX* and the *phn* gene cluster, are only present on a few other cyanobacterial genomes, which suggests environmental or niche-specific adaptation of P metabolism. Databases such as NCBI and IMG now contain complete genome sequences of cyanobacteria with diverse physio-

logical potentials from a range of habitats, e.g., terrestrial and marine, unicellular and filamentous, and several nitrogen-fixing organisms. At this point, a full *phn* gene cluster (encoding both the transporter and C-P lyase) has only been identified in the genomes of the filamentous, heterocyst-forming *Nostoc* strain PCC 7120 and the filamentous, nitrogen-fixing, nonheterocystous *Trichodesmium erythraeum* IMS101 (15), as well as the unicellular, diazotrophic OS-B' *Synechococcus* isolate. All sequenced unicellular marine cyanobacterial genomes contain genes encoding putative Phn transporter components (PhnC, PhnD, and PhnE), but surprisingly, they appear to lack known genes encoding putative C-P lyase subunits (30).

The genes of the *phn* cluster of OS-B' are tightly packed on the genome, and there is significant overlap between adjacent coding sequences. This observation suggests that the environment imposes selective pressure to maintain the operon in a relatively condensed state. It has been suggested that the occurrence of overlapping genes within an operon may be one characteristic of sequences prone to lateral gene transfer (LGT) events (23). The potential for LGT of the *phn* cluster is also consistent with the finding that it is scattered across prokaryotic phylogenies and, moreover, that *phn* genes are not universally present within the cyanobacterial lineage (15). The potential for LGT of *phn* genes is also corroborated by the finding that while *phn* genes are not present on the OS-A genome, the genes flanking the *phn* cluster in OS-B' are syntenic on the OS-A genome; this observation suggests a relatively recent loss or gain of the *phn* cluster. Furthermore, preliminary observations (M. Davison and D. Bhaya, unpublished data) suggest that there is an uncharacterized subpopulation of OS-A that contains the *phn* genes. Additional sequencing of environmental samples from Octopus Spring and Mushroom Spring will likely provide new information concerning the distribution and diversity of *phn* genes in the microbial mat community of the hot springs.

Unlike the *phn* operon of *E. coli*, the OS-B' gene cluster does not contain *phnF*, *phnN*, *phnO*, or *phnP*. PhnF is thought to be a regulatory factor that controls *phn* gene transcription, while PhnN functions in the formation of ribose-phosphate, perhaps an intermediate in some aspect of Phn metabolism (14). The function of PhnO and PhnP are less clear. None of these "missing" genes is required for C-P bond cleavage; they most likely regulate the uptake and/or metabolism of Phns (28). Despite low AAIDs to other Phn polypeptides and the absence of some of the *E. coli* *phn* genes on the OS-B' genome, the OS-B' *phn* gene cluster likely functions in Phn assimilation, since the transcripts accumulate in response to P deprivation and the cells can grow with MePhn as the sole P source, albeit after a long acclimation period. The lack of the putative *phn* regulatory factors in OS-B' raises questions about mechanisms associated with controlling Phn uptake and assimilation in cyanobacteria of the microbial mats. We observed that the transcripts from the second *phn* gene cluster are strongly up-regulated under $-P$ conditions. However, it is not absolutely clear if these transporters are responsible for the uptake of MePhn or other substrates. It has been suggested that the long and variable acclimation phase during which cells grow slowly when Phn is supplied as a sole source of P may be the consequence of steps that are limiting in Phn degradation, particularly transport systems that may take varied amounts of time to

ready for the transport of different Phn compounds (20). Since OS-B' has the potential to synthesize multiple putative Phn transporters, it is necessary to determine the substrate specificities and efficiencies of the various transporters and the extent to which they accumulate in the cytoplasmic membrane.

Even though *phn* transcripts of OS-B' rapidly accumulate in response to P starvation, OS-B' cannot effectively use Phn as a sole P source until the cells have acclimated for approximately 3 weeks. The initial addition of MePhn into the medium suppresses activation of Pho regulon genes that are typically transcribed when P is absent from the medium and also blocks continued cell division. Furthermore, cells that are exposed to MePhn appear to have an exacerbated starvation response, as indicated by reduced cell growth and their increased tendency to bleach and accumulate carotenoids, as monitored by whole-cell absorbance spectra. The negative effects of the MePhn on OS-B' did not result in significant cell death (as visualized via the Live/Dead BacLight system) (data not shown). In cells for which MePhn was the sole source of P, cellular growth arrest and chlorosis was followed by a second phase in which there was steady and slow growth. After about 400 h (~20 days), the cells began to grow more rapidly. This result was potentially a consequence of abiotic degradation of MePhn (although this compound is extremely stable). This explanation was eliminated by demonstrating that once the cells had acclimated, upon transfer into fresh medium containing MePhn as a sole source of P they initiated growth immediately and attained a doubling time similar to that of cells using P_i as their sole P source. We attempted to grow Phn-acclimated OS-B' cells on other Phn substrates, including AEPPhn and glyphosphate, but consistent growth was only observed with MePhn. However, recent preliminary experiments have demonstrated that cells can also grow on EthPhn and do not appear to require an extended acclimation period, as observed with MePhn. A requirement for an extended acclimation period has been observed for *E. coli*; the metabolism of MePhn and cell growth is much delayed relative to the rapid accumulation of the *phn* transcripts (50). The *E. coli* K-12 strain is cryptic for MePhn utilization, and variants that can grow with MePhn as the sole P source are selected for after exposure to the Phn substrate followed by a relatively long lag phase, similar to the situation for OS-B'. The basis for this extended lag period of OS-B' is not known, although in *E. coli*, the lag phase represents the time that it takes to generate a slip strand event in the *phnE* gene; this lesion deletes one of three direct repeat sequences, thereby restoring the function of a membrane component of the Phn transporter (16). The OS-B' MePhn acclimation phenomenon could also represent a genetically based mechanism, as recent growth results indicate that acclimation requires the MePhn substrate presence in addition to P_i starvation and that acclimation to the substrate is not lost over a prolonged period following growth of the cells on P_i in the absence of MePhn (data not shown). In the case of OS-B', it is not clear whether the entire cell population acclimates or whether a subpopulation becomes responsive during the lag phase and ultimately outgrows the cells that were unable to acclimate and utilize Phn. Interestingly, the physiological responses of OS-B' to MePhn suggest that Phn actually inhibits the accumulation of transcripts from genes that comprise the Pho regulon. The reduced ability to respond to P_i starvation when MePhn is

present in the medium indicates that Phns may block transcription of Pho regulon genes (and perhaps other genes as well), either directly or indirectly. For example, it is possible that MePhn interacts with the P_i transport system and thereby suppresses the signaling that is elicited by transport components that work in conjunction with the PhoB/PhoR regulatory elements.

In conclusion, we have investigated how OS-B', a thermophilic cyanobacterium recently isolated from hot springs in Yellowstone National Park, responds to P_i limitation, and we have demonstrated that MePhn can serve as a sole source of P for these organisms. This work also describes the extended period of acclimation required for growth of the cells on MePhn. The *phn* gene cluster has been found in divergent microorganisms isolated from a variety of ecosystems, including some in which Phns constitute a substantial fraction of dissolved organic P of the total P pool (7). The capacity to utilize Phns when other sources of P are limiting would confer an adaptive advantage to the OS-B' ecotype in an environment where P_i may be scarce. Indeed, P_i levels in the Octopus Spring effluent channel drop below concentrations required for activation of the Pho regulon as characterized in *E. coli*, although it not known if the available P_i fluctuates on a temporal or spatial scale. Starvation on a daily or seasonal time frame may allow OS-B' to acclimate to low-P conditions, which includes an increased capability for utilizing phosphonates to satisfy the P demand. If fluctuations in the P_i concentration are frequent, the cells may remain in the acclimated state even when availability is elevated over a short time interval. Further investigations are needed to understand the biogeochemical role of Phns in hot spring environments, the distribution and expression of the *phn* genes among the different bacterial ecotypes, and the exact molecular mechanisms associated with the extended acclimation period that precedes efficient utilization of Phns by OS-B'. We have recently developed in situ approaches to identify gene expression patterns directly in microbial mat samples (42, 43). Now that we have a set of genes that can be used as markers for the P status of these thermophilic cyanobacteria, we will be able to monitor the expression of these genes to determine whether transcripts involved in P acquisition and utilization stay constant or fluctuate over the diel cycle, which will shed light on the different environmental cues that might account for any observed fluctuations.

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