

Phosphorus Scavenging in the Unicellular Marine Diazotroph *Crocospaera watsonii*

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Through the fixation of atmospheric nitrogen and photosynthesis, marine diazotrophs play a critical role in the global cycling of nitrogen and carbon. *Crocospaera watsonii* is a recently described unicellular diazotroph that may significantly contribute to marine nitrogen fixation in tropical environments. One of the many factors that can constrain the growth and nitrogen fixation rates of marine diazotrophs is phosphorus bioavailability. Using genomic and physiological approaches, we examined phosphorus scavenging mechanisms in strains of *C. watsonii* from both the Atlantic and the Pacific. Observations from the *C. watsonii* WH8501 genome suggest that this organism has the capacity for high-affinity phosphate transport (e.g., homologs of *pstSCAB*) in low-phosphate, oligotrophic systems. The *pstS* gene (high-affinity phosphate binding) is present in strains isolated from both the Atlantic and the Pacific, and its expression was regulated by the exogenous phosphate supply in strain WH8501. Genomic observation also indicated a broad capacity for phosphomonoester hydrolysis (e.g., a putative alkaline phosphatase). In contrast, no clear homologs of genes for phosphonate transport and hydrolysis could be identified. Consistent with these genomic observations, *C. watsonii* WH8501 is able to grow on phosphomonoesters as a sole source of added phosphorus but not on the phosphonates tested to date. Taken together these data suggest that *C. watsonii* has a robust capacity for scavenging phosphorus in oligotrophic systems, although this capacity differs from that of other marine cyanobacterial genera, such as *Synechococcus*, *Prochlorococcus*, and *Trichodesmium*.

Phytoplankton, including cyanobacteria from the genera *Prochlorococcus*, *Synechococcus*, *Crocospaera*, and *Trichodesmium*, significantly contribute to overall marine primary production and thereby play a key role in the global cycling of carbon (C). Within the cyanobacteria, diazotrophs, such as *Trichodesmium*, are particularly important because of their ability to fix atmospheric nitrogen (N). Although *Trichodesmium* is perhaps the best-known and best-studied marine diazotroph, recent research (18, 31) has highlighted the biogeochemical importance of small, unicellular diazotrophs (3 to 10 μm), such as *Crocospaera watsonii*, which can introduce a substantial fraction of new nitrogen to the euphotic zone in tropical systems where it occurs (3, 5, 9, 18, 31).

Much of our knowledge regarding unicellular diazotrophs, such as *C. watsonii*, has been driven by recent molecular work to assess the expression of *nifH*, a dinitrogenase reductase-encoding gene responsible for N_2 fixation. This work has led to the identification of two open-ocean *nifH* sequence types, group A and group B. These sequence groups phylogenetically cluster with unicellular cyanobacteria (9, 10, 31). Group A *nifH* DNA phylotypes are most closely related to the genus *Cyanotheca*, while group B *nifH* phylotypes are most similar to *Crocospaera watsonii* WH8501 (5, 31). Quantitative PCR to detect *nifH* has found these phylotypes to be abundant and, at times, more abundant than *Trichodesmium* spp. in oligotrophic waters (5). While *Trichodesmium* is typically most abundant in the upper euphotic zone (4), the unicellular diazotrophs which make up groups A and B have been reported to be more

uniformly distributed through the euphotic zone (17, 18). They also differ from *Trichodesmium* in that they fix nitrogen maximally at night, rather than during the day (31). Ultimately, the diversity, abundance, and widespread distribution of unicellular diazotrophs culminate in high rates of N_2 fixation.

The factors that control the growth and N_2 fixation rates of marine diazotrophs have been intensively studied. One of these factors is the bioavailability of phosphorus. Two major ocean biomes, the North Pacific Subtropical Gyre and the Sargasso Sea, have very low inorganic phosphate concentrations (<1 nM in some cases), high levels of dissolved inorganic N:P, and elevated total dissolved N:P (30). Under these conditions the availability of trace concentrations of dissolved inorganic phosphate (DIP) and the bioavailability of the larger but poorly characterized and chemically heterogeneous pool of dissolved organic phosphorus (DOP) could dramatically influence diazotroph production and N_2 fixation. Several recent studies have identified P bioavailability as a possible controlling factor for the physiology of the diazotroph *Trichodesmium* (7, 23); however, P physiology has not yet been examined with cultures or field populations of the unicellular diazotrophs, such as *Crocospaera*.

Marine cyanobacteria have evolved a number of different strategies for survival in low-DIP marine systems, such as the North Pacific Subtropical Gyre and the Sargasso Sea. Two common strategies for survival in low-phosphate environments include the induction of high-affinity phosphate scavenging systems and the up-regulation of enzymes to hydrolyze DOP into phosphate. Perhaps the best characterized of these strategies is high-affinity phosphate transport. For example, the gene cluster *pstSCAB* has been identified in *Synechococcus* sp. strain WH8102 (26) and *Prochlorococcus* spp. (19). These

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TABLE 1. *Crocospaera watsonii* strains examined in this study

<i>C. watsonii</i> strain	Location	Yr isolated	Axenic
WH8501 ^a	Tropical S. Atlantic	NA ^b	Yes
WH0401	Tropical S. Atlantic	2003	Yes
E12	Tropical S. Atlantic	1985	No
WH0002	Hawaii	2000	Yes
H12	Hawaii	2000	No
H13	Hawaii	2000	No
OFF	Hawaii	2000	No

^a Genome strain.^b NA, not available.

genes are up-regulated by P deficiency in *Synechocystis* sp. strain PCC6803 (27). Also, the presence of the PstS protein can be detected in field populations under low-P conditions (24). Notably absent in the marine cyanobacterial genomes examined to date are any low-affinity phosphate permeases (e.g., *pitA*) (19, 26).

Two of the dominant bond classes of oceanic high-molecular-weight DOP are phosphomonoesters and phosphonates (13). The hydrolysis of phosphomonoesters is mediated by enzymes such as alkaline phosphatase (*phoA*), the activity of which is commonly up-regulated by P deficiency in cyanobacteria (22). The transport of phosphonates is mediated by *phnCDE*. This gene cluster is present in all the available *Synechococcus* and *Prochlorococcus* genomes (19) and in all the *Trichodesmium* species examined to date (6). Hydrolysis of phosphonates can be mediated by multiple enzyme systems of different substrate specificities (14). Phosphonate hydrolysis in marine cyanobacteria has not been comprehensively examined, but evidence from *Synechococcus* sp. strain WH8102 (21, 26) growth studies and *Trichodesmium erythraeum* gene expression analysis (6) suggests that some marine cyanobacteria may be able to metabolize exogenous phosphonate compounds.

Despite these general advances in our understanding of P metabolism in marine cyanobacteria, little is known about how unicellular diazotrophs, such as *C. watsonii*, scavenge phosphorus. Here we used a combination of genomic observations and physiological studies to examine P-scavenging strategies of different strains of *C. watsonii*.

MATERIALS AND METHODS

Cell culturing. Axenic *Crocospaera watsonii* WH8501, previously designated *Synechocystis* sp. strain WH8501, and several other *Crocospaera* sp. strains were obtained from John B. Waterbury at Woods Hole Oceanographic Institution (Table 1). Cultures were grown at 27.5°C using a 14:10-h light-dark cycle provided by cool white fluorescent bulbs with 65 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Cultural purity was confirmed by testing for growth of contaminating organisms with a tryptone-fortified medium (2). Triplicate P-replete (+P) cultures were grown in SN medium (28), with a 0.2- μm -filtered Sargasso seawater base and 45 μM K_2HPO_4 . SO medium was prepared as described above without added nitrogen. Low-phosphate (-P) medium was prepared by reducing the SN/SO medium K_2HPO_4 concentration to 1 μM . For growth experiments on organic phosphorus compounds, K_2HPO_4 was omitted from the base medium, and a suite of organic compounds (DL- α -glycerophosphate, AMP, phytic acid, phosphonoacetic acid, phosphonoformic acid, and 2-aminoethylphosphonate) were added to media at final concentrations of 45 μM . Cells used as the inoculum for all treatments were centrifuged initially for 10 min at 7,000 rpm and resuspended in medium without added phosphate to restrict carryover. Growth of all cultures was monitored in triplicate by relative fluorescence with a Turner Designs 10-AU fluorometer.

Gene expression. To examine gene expression patterns, RNA was extracted from cultures with differing P physiology. Replete (+P) samples were extracted at mid-log phase, and P-starved (-P) samples were extracted at the onset of stationary phase. In one experiment, -P cultures were refed to replete phosphate levels (45 μM). In this case RNA was extracted 24 h after the addition of P. Cells from each condition were harvested by filtering 8 ml onto 0.2- μm polycarbonate filters (25 mm), and the RNA was extracted using a RiboPure-Bacteria kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA concentrations were obtained with a NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE) spectrophotometer. Total RNA was reverse transcribed to single stranded cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), and additional reactions for each sample were set up without reverse transcriptase (RT) to ensure the absence of genomic DNA in no-RT controls. PCR primer sets for a putative alkaline phosphatase (5'AGTGGTGAGGCTGAAGGAGA; 3'ACCCACATTACCTGGAGCAG, gene object identifier = 400846230), *pstS* (5'TTGTGCAACTCAACACAGCA; 3'TTGGATCATTCCAGTTGGT, gene object identifier = 400876370), and a putative phosphate permease (5'CCCCATCTTTTGTAGTTTGA; 3'CAACTAA TGCACCCACAG, gene object identifier = 400856070) were designed based on the sequenced genome of *C. watsonii* WH8501 (<http://img.jgi.doe.gov/pub/main.cgi>; Taxon object identifier = 400330000). Amplicon sizes are noted in Table 2. The PCR primers used to amplify 16S rRNA were the prokaryote-specific 515F (5'GTGCCAGCMGCCGCGGTAA) and 907R (3'CCGYCAATTCMTTTR AGTTT), which yielded a 392-bp product. PCR was performed using 0.2 ng cDNA in a final reaction mixture (15 μl) containing 1 \times PCR buffer (Bio-Rad), 0.25 mM deoxynucleoside triphosphates (Bio-Rad), 0.3 μM of each gene-specific 5' and 3' primer, and 1 unit of *Taq* DNA polymerase (Bio-Rad). Reactions were cycled with an iCycler (Bio-Rad) using a temperature profile of 95°C for 5 min, 95°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min (30 cycles), and a final extension of 72°C for 7 min. Annealing temperatures for the putative alkaline phosphatase, *pstS*, the putative phosphate permease, and 16S rRNA were 58°C, 59.5°C, 61.3°C, and 47°C, respectively. PCR products were resolved on a 2% agarose gel, stained with ethidium bromide, and imaged with a Gel Logic 440 imaging system (Kodak, Rochester, NY). The results were semi-quantified by normalizing the band intensities to those obtained from the 16S rRNA gene using 1D image analysis software (Kodak), allowing for assessments of the presence or absence of the target gene but not relative abundance.

Sequencing. Genomic DNA extracts for several *C. watsonii* strains were obtained from I. Ehrenreich (Table 1). The extraction procedure is described elsewhere (8). PCR amplification of each gene amplicon was conducted using 1 μl of each DNA extract (diluted 1:100) with primer sequences and reaction conditions as detailed above. The putative alkaline phosphatase, *pstS*, and the putative phosphate permease fragments were identified and gel purified using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). Sequencing of PCR products was performed at either the sequencing facility at the Bay Paul Center at the Marine Biological Laboratory, Woods Hole, MA, or at the University of Maine, Orono, ME. All sequence data were analyzed manually using the programs Sequencher (Gene Codes Corporation, Ann Arbor, MI), MacVector (Accelrys, Burlington, MA), and BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST>).

Genome observations. Genomic topology was visualized with the publicly available Integrated Microbial Genomes (IMG) interface on the Department of Energy-Joint Genome Institute web page (<http://img.jgi.doe.gov/cgi-bin/pub/main.cgi>). Homology searches were also performed using this interface. Genes were assigned a putative function or gene name based on percent identity over at least 80% of the open reading frame (ORF). In two cases, the gene was annotated based on 60% of the ORF where the topology supported the gene call. The evidence for these annotations and the complete sequences can be viewed with the IMG gene object identifier (Table 2) at the above URL.

Nucleotide sequence accession numbers. *C. watsonii* sequences determined in this study have been deposited in GenBank with the following accession numbers: putative alkaline phosphatase, DQ297912 to DQ297914; putative phosphate permease, DQ297915 to DQ297920; and *pstS*, DQ297921 to DQ297924.

RESULTS

Genome observations. Observations from the *C. watsonii* WH8501 genome suggest that this diazotroph may have both low- and high-affinity phosphate transport systems. A putative low-affinity phosphate permease is present next to a putative copy of the phosphate transport regulator *phoU* (Table 2). A complete cluster of genes encoding the high-affinity transport

TABLE 2. Putative phosphorus scavenging genes present in the *Crocospaera watsonii* WH8501 genome

Category	IMG gene object identifier	Putative function	% Similarity to reference	Reference	
Low-affinity phosphate transport	400856070	Phosphate permease (transporter) low affinity	51.96	<i>Shewanella frigidimarina</i> NCMIB400	
Unknown function	400885240	PhoU family—involved in phosphate transport	65.45	<i>Synechocystis</i> sp. strain PCC6803	
	400856060	PhoU family—involved in phosphate transport	27.07	<i>Shewanella frigidimarina</i> NCMIB400	
	400872090	Phosphate starvation-inducible protein, <i>phoH</i>	76.73	<i>Synechocystis</i> sp. strain PCC6803	
Hydrolysis of phosphate esters	400846230	Alkaline phosphatase ^a	27.44	<i>Deinococcus geothermalis</i> DSM11300	
	400841270	Phosphoesterase	38.1	<i>Synechocystis</i> sp. strain PCC6803	
	400869600	Metallophosphoesterase	53.7	<i>Trichodesmium erythraeum</i> IMS101	
	400867140	Alkaline phosphatase-like, <i>dedA</i>	59.61	<i>Synechocystis</i> sp. strain PCC6803	
High-affinity phosphate transport	400890860	Phosphate binding, <i>pstS</i>	33.33	<i>Methanothermobacter thermautotrophicus</i> Delta H	
	400876370	Phosphate binding, <i>pstS</i>	65	<i>Synechocystis</i> sp. strain PCC6803	
	400878470	Phosphate binding, <i>pstS</i>	50.45	<i>Synechocystis</i> sp. strain PCC6803	
	400876380	Phosphate permease, <i>pstC</i>	57.63	<i>Synechocystis</i> sp. strain PCC6803	
	400876390	Phosphate permease, <i>pstA</i>	29.82 ^b	<i>Methanosarcina mazei</i> Gol	
	400876400	Phosphate permease, <i>pstA</i>	69.23 ^b	<i>Synechocystis</i> sp. strain PCC6803	
	400876410	ATPase component, <i>pstB</i>	76.78	<i>Synechocystis</i> sp. strain PCC6803	
	400876420	ATPase component, <i>pstB</i>	64.23	<i>Synechocystis</i> sp. strain PCC6803	
	Polyphosphate metabolism	400890460	Polyphosphate kinase, <i>ppK</i>	74.86	<i>Synechocystis</i> sp. strain PCC6803
		400849750	Exopolyphosphatase, <i>ppX</i>	62.84	<i>Synechocystis</i> sp. strain PCC6803
400852200		Inorganic pyrophosphatase, <i>ppA</i>	82.71	<i>Nostoc punctiforme</i> PCC73102	

^a Although much smaller, this gene is 48.46% similar to the atypical alkaline phosphatase characterized in *Synechococcus* PCC7942 (22).

^b Percent similarity over 60% of the ORF; all other similarities reported are over 80% of the ORF.

of phosphate (*pstSCAB*) are also present in the *C. watsonii* WH8501 genome (Table 2). There are multiple, unique *pstS* orthologs distributed across different contigs, although the genes 400876370 and 400878470 are immediately adjacent to a complete phosphate transport system (*pstABC*) (Fig. 1). In this gene cluster, there are duplications of full-length *pstB* and truncated *pstA* (Fig. 1).

One common P stress strategy is to up-regulate enzymes that will hydrolyze DOP into bioavailable phosphate. There are no clear homologs of the *phnCDE* phosphonate transport system that is present in *Synechococcus* WH8102 (SYNW1168, 1169, 1170 at http://genome.ornl.gov/microbial/syn_wh/). There are also no apparent homologs of *phnWX*, the genes thought to encode for phosphonate hydrolysis in *Synechococcus* WH8102 (26). Further, there are no homologs of the *phn* gene cluster that encode the transport and hydrolysis of phosphonates in the diazotroph *T. erythraeum* (6). In contrast, *C. watsonii* ap-

pears to have a broad spectrum of genes with a putative role in phosphomonoester hydrolysis (Table 2).

Three genes with a potential role in polyphosphate metabolism were identified (Table 2). The putative *ppK* gene encodes a polyphosphate kinase for the synthesis of polyphosphate (15). The putative *ppX* gene encodes an exopolyphosphatase that sequentially releases phosphate from long-chain polyphosphate (11, 15). A third putative gene, *ppA*, encodes a soluble inorganic pyrophosphatase (11). None of these ORFs are contiguous in the current assembly of the *C. watsonii* WH8501 genome.

Gene expression. The expression of *pstS*, the putative phosphate permease, and the putative alkaline phosphatase were examined in axenic cultures of *C. watsonii* WH8501 grown under different conditions. Expression of *pstS* was detected in -P cultures grown as described above but not in +P cultures (Fig. 2). No *pstS* expression was detected in a -P culture refed (PR) to replete phosphate levels (Fig. 2). The expression of the putative phosphate permease was detected both in -P cultures grown as described above and in +P cultures (Fig. 3). To date, no consistent expression of the putative alkaline phosphatase could be detected. The 16S rRNA expression was very consistent across all treatments in both experiments (Fig. 2 and Fig. 3) and infrequently required normalization.

Strain comparison. Strains isolated from both the Pacific (WH0002, H12, H13, and OFF) and the Atlantic (WH0401 and E12) were examined for the presence of *pstS*, the putative phosphate permease, and the putative alkaline phosphatase. The putative alkaline phosphatase and *pstS* genes were amplified and sequenced from strains OFF (*pstS* only), WH0401,

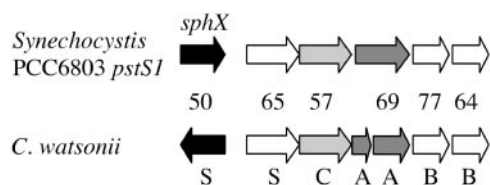


FIG. 1. Topology of the genes encoding high-affinity phosphate transport in *Crocospaera watsonii* and *Synechocystis* PCC6803. The genes *pstS*, -A, -B, and -C are designated with single letters; *sphX*, a member of the same gene family as *pstS* (1, 16), is also designated. Shading indicates the genes being compared with the percent similarity of each pair.

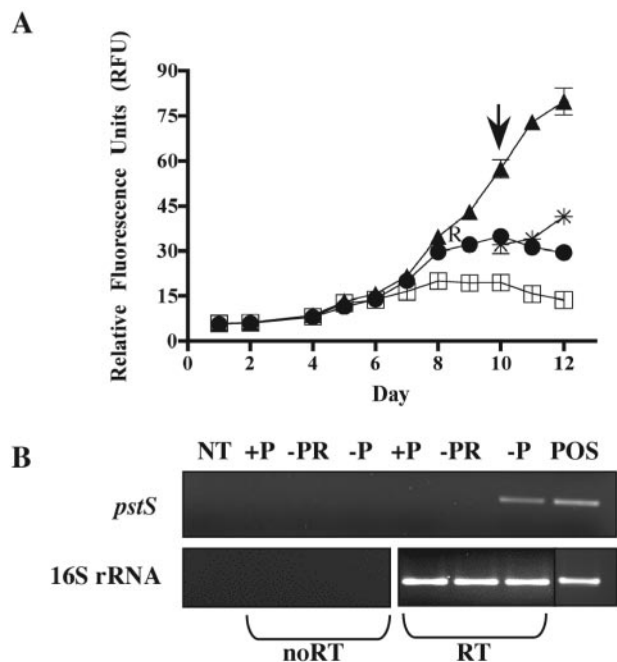


FIG. 2. Expression of *pstS* (400876370). (A) A growth curve, plotted as relative fluorescence units, of axenic *Crocospheara watsonii* WH8501 in SN medium with 45 μ M (triangle), 1 μ M (circle), or no added (square) phosphate. The "R" indicates where the 1 μ M culture was refed (*) to 45 μ M. Arrow indicates where samples for gene expression were harvested. Error bars denote the standard error from the mean ($n = 3$). (B) Expression of the 289-bp *pstS* amplicon and the 392-bp 16S rRNA amplicon. Lane designations are as follows: NT (no template), +P (cDNA from a P-replete culture, harvested as designated), -PR (cDNA from a P-starved culture refed to replete levels of P, harvested as designated), -P (cDNA from a P-starved culture, harvested as designated), POS (DNA from a P-replete culture). Samples transcribed without or with reverse transcriptase are designated noRT or RT, respectively.

H12, and WH0002 (3). Sequence alignments of the *pstS* and *phoA* gene fragments showed high sequence similarity to strain WH8501, with no consistent differences between Pacific and Atlantic isolates (Table 3). Nucleotide sequence alignments comparing *C. watsonii* WH8501 *pstS* to the *Synechococcus* WH8102 and *Trichodesmium* IMS101 genes were 27% and 35% similar, respectively (data not shown). A series of strains was also examined for the putative low-affinity phosphate transporter, and all the strains tested have the gene. Sequence identity across the 294-bp amplicon was 99 to 100% (Table 3).

Growth studies. Axenic cultures of *C. watsonii* WH8501 (Atlantic isolate) and WH0002 (Pacific isolate) were able to grow on a variety of phosphomonoesters as a sole source of added phosphorus. Specifically, *C. watsonii* WH8501 growth occurred on phytic acid, AMP (Table 4), and glycerophosphate (Fig. 4). Conversely, there was no growth relative to the control (no P added) on the phosphonate, phosphonoformic acid (Fig. 4), and there has been no growth observed on any phosphonate compounds tested to date (Table 4). Of the compounds tested, similar growth patterns were observed for *C. watsonii* WH0002 (Table 4). Further, growth patterns were similar with (SN) or without (SO) an N source added to the medium (data not shown).

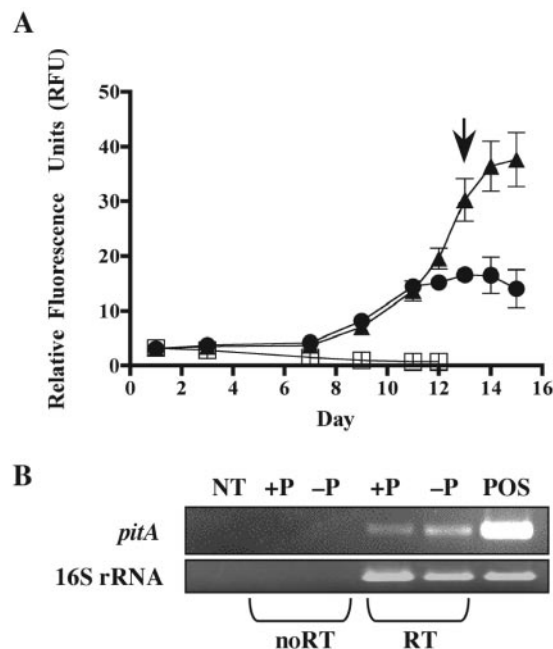


FIG. 3. Expression of a putative phosphate permease (400856070). (A) A growth curve, plotted as relative fluorescence units, of axenic *Crocospheara watsonii* WH8501 in SN medium with 45 μ M (triangle), 1 μ M (circle), or no added (square) phosphate. Arrow indicates where samples for gene expression were harvested. Error bars denote the standard error from the mean ($n = 3$). (B) Expression of the 294-bp putative phosphate permease amplicon (indicated as *pitA*) and the 392-bp 16S rRNA amplicon. Lane designations are as follows: NT (no template), +P (cDNA from a P-replete culture, harvested as designated), -P (cDNA from a P-starved culture, harvested as designated), POS (DNA from a P-replete culture). Samples transcribed without or with reverse transcriptase are designated noRT or RT, respectively.

DISCUSSION

The factors that constrain nitrogen fixation in the open ocean remain a topic of intense study and debate among oceanographers. Several recent studies have highlighted the importance of phosphorus to the growth and nitrogen fixation rates of the diazotroph *Trichodesmium* (7, 23), but little is known about the phosphorus physiology of the unicellular photosynthetic diazotrophs, such as *C. watsonii*, and the extent to

TABLE 3. Percent similarities of gene sequences related to P scavenging recovered from various *Crocospheara watsonii* strains to orthologous sequences from *C. watsonii* WH8501

Strain	% Similarity to WH8501 sequence ^a		
	<i>pstS</i> (289 bp)	Putative alkaline phosphatase gene (246 bp)	Putative phosphate permease gene (294 bp)
WH0401	100	98	100
E12	100	‡	99
WH0002	100	98	100
H12	100	99	100
H13	‡	‡	100
OFF	100	‡	100

^a Amplicon sizes are given in parentheses. ‡, gene fragment did not amplify.

TABLE 4. Phosphorus sources used for growth studies of *Crocospaera watsonii*

Phosphorus source	Abbreviation	Bond class	Growth	
			WH8501	WH0002
DL- α -Glycerophosphate	GP	C-O-P	Yes	Yes
Adenosine monophosphate	AMP	C-O-P	Yes	Yes
Phytic acid	PA	C-O-P	Yes	ND ^a
Phosphonoacetic acid	PN	C-P	No	ND
Phosphonoformic acid	PFA	C-P	No	ND
2-Aminoethylphosphonate	2-AEP	C-P	No	No

^a ND, not determined.

which phosphorus bioavailability may influence growth and nitrogen fixation in this group.

Using the publicly available sequence of the *C. watsonii* WH8501 genome, we identified gene homologs related to P metabolism, including genes related to the metabolism of polyphosphate, phosphate transport, and the hydrolysis of DOP. The presence of genes encoding proteins involved in the synthesis (e.g., *ppK*) and degradation (e.g., *ppX*) of polyphosphate in *C. watsonii* WH8501 is consistent with the presence of these genes in the other marine picocyanobacteria (19). Some strains of *Prochlorococcus* and *Synechococcus* can grow on polyphosphate as a sole source of P (19): as such, polyphosphate could be an important P source for *C. watsonii* in oligotrophic systems.

High-affinity phosphate transport may be a key adaptation of marine cyanobacteria that allows them to persist in low-environmental-DIP conditions. In the marine cyanobacterial genomes examined to date, multiple distinct *pstS* sequences, encoding the high-affinity phosphate binding protein PstS, have been identified (19, 27). This is also the case with *C. watsonii*, where there are three copies of *pstS*. Two of these copies of *pstS* are clustered together with a complete transport complex (*pstABC*), which has a duplication in both *pstB* and *pstA*. These *pst* genes are similar to genes of *Synechocystis* sp. strain PCC6803, which also has a duplication in *pstB*. *Synechocystis* sp. strain PCC6803 has two high-affinity phosphate transport systems, Pst1 and Pst2 (27), and the *C. watsonii* WH8501 complex is most similar to the Pst1 system. In microarray-based regulation studies, the Pst1 system was induced more rapidly than the Pst2 system in response to P stress (27). Although we have not examined the timing of expression in the different copies of *pstS* or the different components of the *pstABC* system, one of the *pstS* genes in this cluster (400876370) is clearly regulated by P supply in culture. This P regulation is consistent with studies in *Synechocystis* PCC6803 (27).

The expression of the PstS protein has been used as a target for assays of P stress in field populations of marine *Synechococcus* and *Prochlorococcus* (24). Considering the strong P regulation of *C. watsonii* WH8501 *pstS* expression, it may be possible to use this gene as a target for RT-PCR assays of P physiology. Although the sequence is identical over our amplicon in different *C. watsonii* strains, there is substantial sequence heterogeneity between *C. watsonii* and the *pstS* sequences of other picocyanobacteria, suggesting that gene expression studies in the field should be able to distinguish *C. watsonii* sequences from those of other cyanobacteria. RT-

PCR assays of *C. watsonii* *pstS* may be used to examine the extent to which P supply constrains the growth and nitrogen fixation rates of *Crocospaera* in the field.

One of the more striking, and unexpected, observations from the *C. watsonii* WH8501 genome is the presence of a putative low-affinity phosphate permease. *C. watsonii* has an ORF of 372 amino acids that is 25% similar to the 5' region of the 499-amino-acid *pitA* gene involved in phosphate transport in *Escherichia coli* (reference 12 and references therein). The *C. watsonii* ORF is also 51% similar to a gene encoding a 347-amino-acid product in *Shewanella frigidimarina* (NCMIB400) that is annotated as a phosphate transporter, although it has not been experimentally characterized. No homologs of *pitA* or low-affinity phosphate permeases have been identified in the marine cyanobacterial genomes examined to date (19, 26), and others have suggested that the lack of *pitA* in the other marine cyanobacterial genomes is because the K_m of *E. coli* *pitA* would not be efficient in marine systems, where DIP is often quite low (26). Yet we have been able to identify the gene in all of the *C. watsonii* strains we have tested. In addition, the putative phosphate permease gene is expressed in phosphate-replete axenic cultures of *C. watsonii* WH8501. Also of note is the presence of two putative copies of *phoU*, a modulator of inorganic phosphate transduction, which, like the putative phosphate permease, is not detectable in any of the marine picocyanobacteria with published genomes (19). The widespread presence of the putative phosphate permease gene in *Crocospaera* and its expression suggest that this gene is serving a function for this genus. Further work is warranted to determine if the gene is indeed being used for the low-affinity transport of phosphate.

Phosphorus bioavailability is in part determined by the presence or absence of P scavenging systems within a cell, and genomic observations suggest that *C. watsonii* strains have a robust capacity to hydrolyze phosphomonoesters in the marine DOP pool. Genome annotation identified at least four genes that appear to encode phosphomonoesterases. These genomic observations are consistent with data presented herein, where axenic *C. watsonii* WH8501 was able to grow on a variety of phosphomonoesters as a sole source of added phosphorus. We were unable to consistently detect expression of the putative

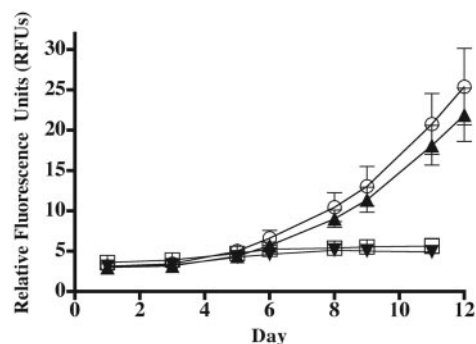


FIG. 4. A representative growth curve, plotted as relative fluorescence units, of axenic *Crocospaera watsonii* WH8501 in SN medium with 45 μ M phosphate (triangle: potassium diphosphate), 45 μ M phosphomonoester (circle: glycerophosphate), 45 μ M phosphonate (reverse triangle: phosphonoformic acid), or no phosphate (square) added. Error bars denote the standard error from the mean ($n = 3$).

alkaline phosphatase (400846230) that we examined, and it may be that one or more of the other putative phosphomonoesterases listed in Table 2 are mediating this organism's growth on this bond class of DOP.

Homologs of *phnCDE*, encoding proteins involved in phosphonate transport, have been documented in numerous marine cyanobacteria including *Synechococcus* sp. strain WH8102 (19, 21, 26), *Prochlorococcus* sp. strain MIT9313 (19), and *T. erythraeum* IMS101 (6). Although there are many ABC transporters in the *C. watsonii* genome, we are unable to identify clear homologs of *phnCDE*. Phosphonate hydrolysis in heterotrophic bacteria (e.g., *Pseudomonas stutzeri*) is typically controlled by a suite of *phn* genes (*phnF* to *-P*) that encode a C-P lyase complex (29). The *phnF* to *-P* genes of the C-P lyase complex have not been identified in any of the picocyanobacterial genomes examined to date (19). However, *Synechococcus* sp. strain WH8102 will grow on the phosphonates 2-aminoethylphosphonate and ethylphosphonate, and here phosphonate hydrolysis is thought to be mediated by a different mechanism (putatively *phnWX*), since the genes *phnF* to *-P* are absent (26). In *C. watsonii*, there are no clear homologs of either *phnF* to *-M* or *phnWX*, suggesting this genus may be lacking in the ability to metabolize exogenous phosphonates as a phosphorus source. Growth studies corroborate these genomic observations in that growth for two strains was observed on phosphomonoesters but not on any of the phosphonates tested to date. This apparent lack of phosphonate metabolism is particularly striking relative to the diazotroph *T. erythraeum*, which has a complete C-P lyase complex, likely allowing this organism to metabolize a variety of exogenous phosphonate compounds (6).

Genes potentially involved in phosphorus scavenging (e.g., phosphate transport and DOP hydrolysis) have been described for several genera of marine cyanobacteria, including *Prochlorococcus* spp. (19, 25) and *Synechococcus* spp. (19, 26). These studies and our ongoing research with *Trichodesmium* spp. (20) have highlighted that there can be substantial heterogeneity in the presence and regulation of P-scavenging genes in different genera, and even between the presence and absence of these genes in strains of the same genus. For example, the genome of *Prochlorococcus* sp. strain MED4 has a gene encoding a putative alkaline phosphatase (*phoA*), whereas *Prochlorococcus* sp. strain MIT9313 does not (19, 25). Genes putatively encoding a high-affinity phosphate binding protein (*pstS*) and an alkaline phosphatase were examined in multiple *C. watsonii* strains isolated from different years and from both the Pacific and the Atlantic. The *pstS* sequence is identical over the 289-bp amplicon in all the strains examined. The putative alkaline phosphatase gene is 98 to 99% similar over the 246-bp amplicon. Although strains from the Atlantic as well as the Pacific appear to have these two P-scavenging-related genes, there are a few strains that we have been unable to amplify using the primer sets reported here. The reasons for this are unclear, but they hint at the possibility of sequence divergence or potentially the absence of these genes in some strains.

There is a growing body of research on unicellular diazotrophs, such as *Crocospaera*, although much of this work has been focused on their distribution and N physiology. Our data suggest that this genus shares some of the adaptations to low DIP commonly found in marine cyanobacteria, including

the capacity for the high-affinity transport of phosphate and the hydrolysis of phosphomonoesters. Despite these similarities, our data also suggest some fundamental differences between *Crocospaera* and other marine cyanobacteria, including the apparent lack of phosphonate utilization and the presence of a putative low-affinity phosphate permease. If these differences are borne out in field populations, *Crocospaera* occupies a different niche with regard to competition for P than other marine cyanobacteria.

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