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ORIGINAL ARTICLE

Alternative pathways for phosphonate metabolism in thermophilic cyanobacteria from microbial mats

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Synechococcus **sp. represents an ecologically diverse group of cyanobacteria found in numerous environments, including hot-spring microbial mats, where they are spatially distributed along thermal, light and oxygen gradients. These thermophiles engage in photosynthesis and aerobic respiration during the day, but switch to fermentative metabolism and nitrogen fixation at night. The genome of** ***Synechococcus*** **OS-B′, isolated from Octopus Spring (Yellowstone National Park) contains a** ***phn*** **gene cluster encoding a phosphonate (Phn) transporter and a C–P lyase. A closely related isolate,** ***Synechococcus*** **OS-A, lacks this cluster, but contains genes encoding putative phosphonatases (Phnases) that appear to be active only in the presence of the Phn substrate. Both isolates grow well on several different Phns as a sole phosphorus (P) source. Interestingly,** ***Synechococcus*** **OS-B′ can use the organic carbon backbones of Phns for heterotrophic growth in the dark, whereas in the light this strain releases organic carbon from Phn as ethane or methane (depending on the specific Phn available);** ***Synechococcus*** **OS-A has neither of these capabilities. These differences in metabolic strategies for assimilating the P and C of Phn by two closely related** ***Synechococcus*** **spp. are suggestive of niche-specific constraints in the evolution of nutrient assimilation pathways and syntrophic relationships among the microbial populations of the hot-spring mats. Thus, it is critical to evaluate levels of various P sources, including Phn, in thermally active habitats and the potential importance of these compounds in the biogeochemical cycling of P and C (some Phn compounds also contain N) in diverse terrestrial environments.**

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Introduction

Phosphorus (P) is essential for cell growth, as it is a vital macromolecular constituent of phospholipids, proteins, polysaccharides and cofactors (Torriani-Gorini *et al.*, 1994). Because phosphate minerals are sparingly soluble ($[PO_4^{3}] = 1\,\mu M$ at pH 7, 25 °C), and the geochemical cycling of phosphate is slow, the concentration of orthophosphate is typically the major limiting nutrient in both marine and terrestrial environments (Wu *et al.*, 2000; Mills *et al.*, 2004; Elser *et al.*, 2007). Thus the ability to use alternate P sources is an advantage. For instance, phosphonates (Phns) appear to constitute a significant fraction of dissolved organic P in the oceans. In parts of the Pacific Ocean, Phns can constitute as much as 25% of the dissolved, high-molecular-weight organic P (Clark *et al.*, 1998; Kolowith *et al.*, 2001) although the exact source of the Phns is not known. Phns can be components of membrane phosphonolipids, nucleic acids, proteins and polysaccharides (Kononova and Nesmeyanova, 2002).

Phn use by microbes may be an important and potentially underestimated component of P biogeochemical cycling (Benitez-Nelson *et al.*, 2004; Falkowski *et al.*, 2008; Martinez *et al.*, 2009; Van Mooy *et al.*, 2009). Cyanobacteria in marine habitats (Dyhrman *et al.*, 2006, 2009; Sanudo-Wilhelmy, 2006; Karl *et al.*, 2008; Ilikchyan *et al.*, 2009; Orchard *et al.*, 2009) such as *Trichodesmium* sp. encode C–P lyases that allow them to use Phns as a P source and as much as 10% of the total internal P can exist as Phns (Dyhrman *et al.*, 2006, 2009). Phns are widely found in terrestrial environments and anthropogenic sources of Phns are ubiquitous. Various Phns are widely used as pesticides, detergent additives, antibiotics and flame retardants (Kononova and Nesmeyanova, 2002; Nowack, 2003; Singh, 2009).

We have obtained complete genome sequences for two related *Synechococcus* isolates from microbial mats in Octopus Spring, which is located in the Lower Geyser Basin of Yellowstone National Park: *Synechococcus* OS-A (*Syn* OS-A) and OS-B′ (*Syn* OS-B′) (Bhaya *et al.*, 2007). These isolates are

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differentially distributed along the horizontal thermal gradient of the mat. *Syn* OS-B′ is prevalent at 53–60 °C whereas *Syn* OS-A dominates at higher temperatures (58–65 °C) (Allewalt *et al.*, 2006; Kilian *et al.*, 2007). In recent studies we showed that both *Syn* OS-A and *Syn* OS-B′ contain an extensive suite of genes in the Pho regulon, which is required for efficient acquisition of P (Supplementary Table S1). This includes a putative operon with 14 genes encoding proteins for phosphonate (Phn) transport (encoded by *phnCDE*) and C–P lyase activity (encoded by *phnG-M*), which is present in the *Syn* OS-B′ genome but lacking in *Syn* OS-A (Adams *et al.*, 2008). In this study we have examined the ability of the two closely related cyanobacterial isolates, *Syn* OS-A and *Syn* OS-B′, to grow on and use different Phn sources, which suggests that there are alternative strategies for metabolizing Phn in the microbial mats. In a complementary approach, we also monitored changes in expression of the cyanobacterial C–P lyase and phosphonatase (Phnase) genes *in situ* over a diel cycle.

Material and methods

Culture conditions

Axenic cultures of *Syn* OS-A (CIW 14) and *Syn* OS-B′ (CIW 10) were grown as previously described (Adams *et al.*, 2008). Axenic cultures of *Syn* OS-A (CIW 14) were isolated from original enrichment cultures using the same strategy as described by Kilian *et al.* (2007). Cultures were grown and maintained at 50 °C in liquid D medium supplemented with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.2–8.3)) and Va vitamins. This growth medium, termed DH10, contains Pi as sodium phosphate at a concentration of 0.77 mM. Pi-free medium (DH10–P) was prepared by replacing the sodium phosphate with an equimolar amount of sodium chloride. Cultures were bubbled with a 3% CO_2 in air mixture in a 50 °C incubator, under continuous light ($\sim$75 μmol photon m^{-2} s^{-1}).

Cells were grown in DH10–P medium supplemented with 0.5 mM methylphosphonate (MePhn), ethylphosphonate (EtPhn) or aminoethylphosphonate (AePhn) obtained from Sigma-Aldrich (St. Louis, MO, USA; MePhn 15730HG, AePhn, 268674–1G and EtPhn, 289876-1G, grade purum). Experiments with *Syn* OS-B′ were performed with cells acclimated to growth on Phn, as previously described (Adams *et al.*, 2008). *Syn* OS-A cells were not subjected to an acclimation period.

In situ *sample collection, extraction and qRT-PCR*

Samples were collected on 28–29 July 2007, from the effluent channels of Octopus Spring at Yellowstone National Park (latitude 44.5340836, longitude −110.7978895) (Steunou *et al.*, 2008). Frozen mat core samples or cells were processed for RNA or DNA extraction as described previously (Steunou *et al.*, 2006, 2008). Briefly, samples for DNA extraction were collected using cork borers to remove an entire core/plug from the mat. Cores were separated into the top green layer (typically between 1 and 3 mm) and the bottom orange layer (typically 2 and 4 mm). The top green layer contains the majority of the cyanobacteria and was used for nucleic acid extraction. Immediately after collection, samples were frozen in liquid nitrogen to prevent nucleic acid degradation.

Genes within the *phn* operon were amplified from mat samples using specific primers (confirmed by sequencing). Reverse transcription of RNA samples was performed as described previously (Adams *et al.*, 2008). For all experiments a 'No reverse transcriptase added' control was carried out. Specific primers were used to amplify segments ($\sim$200 bp) from all cDNAs measured (Supplementary Table S2).

For gene expression analyses (Figure 4), we took cells from cultures bubbled with a 3% CO_2 in air mixture in a 55 °C incubator, under continuous light ($\sim$75 μmol photon m^{-2} s^{-1}). Cells were collected during exponential phase (day 5) after starting a culture at an OD 750 of 0.05. Three biological replicates, each with three technical replicates, were performed to calculate standard error. The level of expression of each gene, when the cells were grown under +P conditions, was considered the control. The rest of the expression values were normalized based on this control, which was taken as 1. For the *in situ* experiment (Figure 5), we had access to only one biological replicate for each time point (see above for sample processing), but three technical replicates were performed. We determined the absolute value of the RNA encoding a specific gene, among all environmental samples over the diel cycle using primers specific for CYA_1475, CYA_2058 and CYB_0159 (*phnC*) (Supplementary Table S3). We normalized these values based on the smallest value over the diel cycle for each gene (arbitrarily set at 1). Data are shown as relative units (RU) (Whelan *et al.*, 2003; Steunou *et al.*, 2006). Primers specific for *Syn* OS-A or *Syn* OS-B′ genes (Supplementary Table S2) were designed using MetaPrime, a Primer3-based oligonucleotide generation program (Rozen and Skaletsky, 2000); specificity at the 3′ end of the primers allows for the generation of variant-specific oligonucleotides.

Methane and ethane measurements

Cultures were maintained in a sealed vessel in either the light (75 μmol photon m^{-2} s^{-1}) or dark for various times and the headspace was sampled. Methane and ethane were resolved on a Porapak N column (3 mm internal diameter × 2 m length) with helium as the carrier gas, and quantified using a Shimadzu (Tokyo, Japan) gas chromatograph (GC-8A) equipped with a flame ionization detector. Temperature settings were 60 °C for the column and 100 °C for both the injector

and the detector. Calibrations were performed with methane- and ethane-in-nitrogen standards (Scotty II Analyzed Gases, 100 p.p.m.).

Bioinformatics
Putative Phnases were identified in *Syn* OS-A and *Syn* OS-B′ by BLAST analyses using previously identified Phnase proteins. Full sequences for known Phnase proteins were obtained from GenBank, and from these, selected sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), hand-edited using the GCG Wisconsin package sequence editor and the Gene.Doc program (http://www.nrbsc.org/gfx/genedoc/).

Results

Growth with Phn as a sole P source
We recently showed that *Syn* OS-B′ can grow using methyl phosphonate (MePhn) as a sole source of P, but growth was contingent on an acclimation period of approximately 3 weeks in the presence of MePhn (Adams *et al.*, 2008). These acclimated cells attained growth rates on MePhn (as the sole source of P) comparable to growth on phosphate (Pi). Consequently, for all growth experiments described here, we used *Syn* OS-B′ cells that had been acclimated to MePhn for at least 3 weeks (Adams *et al.*, 2008). This acclimation requirement has been described for several other microorganisms, but it is not understood at the mechanistic level except in *Escherichia coli* (Iqbal *et al.*, 2004). *Syn* OS-A cells were not initially subjected to an acclimation regime and it appears that they do not require an acclimation period to be able to use Phns.

We tested the ability of *Syn* OS-B′ and *Syn* OS-A to grow on various Phn compounds, including MePhn, EtPhn, phenylphosphonate (PhenylPhn) and AePhn, and the herbicide glyphosate (Hildebrand and Henderson, 1983). To establish maximal growth rates, we grew cells on medium containing Pi, which is generally the preferred P source for microbes. To establish rates of growth when the cells were deprived of P ('minimum' growth rates), we used acclimated *Syn* OS-B′ cells or nonacclimated *Syn* OS-A cells that were starved for P (that is, grown for 1 week, as they undergo 3–4 doublings, on medium lacking all P before the start of the experiment) (Figure 1). This prestarvation was necessary because these cyanobacteria accumulate high levels of polyphosphate (poly P) ($\sim 39 \pm 0.1$ nmol poly P per mg of protein) when grown in P-containing medium.

Both MePhn and EtPhn served as a sole source of P for *Syn* OS-B′, with rates of growth that were comparable to those observed when Pi was used as the sole P source (Figure 1a). *Syn* OS-B′ grew slightly slower with fewer doublings (four doublings instead of five) when AePhn was the sole P source. Neither *Syn* OS-B′ nor *Syn* OS-A could grow when glyphosate was substituted for Pi in the medium, although certain cyanobacteria can use glyphosate as a sole source of both P and N (Forlani *et al.*, 2008). Unexpectedly, *Syn* OS-A cells were also able to maintain high rates of growth when various Phn compounds were provided as a sole source of P (Figure 1b), even though they lack the operon encoding the C–P lyase and Phn transporter. In contrast to *Syn* OS-B′, no acclimation period was required for *Syn* OS-A. Growth occurred immediately after *Syn* OS-A was transferred to medium devoid of Pi but supplemented with Phns. Once the internal Pi reserves were depleted, the cells appeared to be able to access the Phn compounds in the medium as a source of P.

Methane and ethane production
The results described above and comparative genomic analysis raised the possibility that *Syn* OS-A and *Syn* OS-B′ have different mechanisms for the

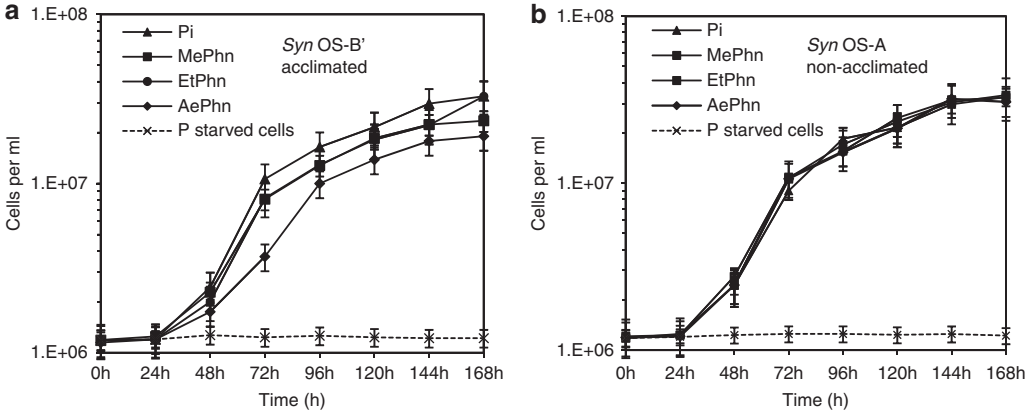

Figure 1 Time course of growth on Phn sources. Growth of acclimated *Syn* OS-B′ (**a**) or *Syn* OS-A (**b**) at 50 °C in complete DH10 medium (Pi), DH10–P (P starved), or DH10–P supplemented with 0.5 mM MePhn, EtPhn or AePhn. Controls show growth of *Syn* OS-B′ or *Syn* OS-A cells maintained in medium lacking P (3 weeks for *Syn* OS- B′ and 1 week for *Syn* OS-A) and then, kept in the same medium over the course of the experiment (minimum growth control). Cells per ml are shown on the *y* axis on a log scale. The P source for each growth curves is given in the figure. The color reproduction of this figure is available on the html full text version of the manuscript.

acquisition of P from Phn. Furthermore, MePhn and EtPhn have the potential to serve as sources of both P and C (Cook *et al*., 1978; Ternan *et al*., 1998), and cells that assimilate the P but not the C moiety of Phns might release methane and ethane into the environment (Yakovleva *et al*., 1998). However, cells that can assimilate the fixed C of the Phn might be able to grow in the dark using Phn as a sole source of reduced carbon. To test these possibilities, we grew *Syn* OS-B′ on either EtPhn or MePhn in continuous light (75 μmol photon m^{-2} s^{-1}) in sealed test tubes, and monitored methane or ethane over a period of 168 h by gas chromatography. Methane and ethane accumulated in the headspace when the sole P source was MePhn and EtPhn, respectively (Figures 2a and b). The gas accumulation tracked cell growth as measured by absorbance at 750 nm. Growth of *Syn* OS-B′ with AePhn as a sole P source in the light also resulted in methane release (no ethane was released), but the final methane concentration achieved in the headspace after 48 h was ~5-fold lower than for MePhn, and the final cell density was ~2-fold less (Figure 2c). As expected, neither methane nor ethane was released by cells growing in medium containing Pi but lacking Phn; medium lacking cells also did not release ethane or methane. These results show a direct correlation between cell growth, which requires the acquisition of P from Phn, and the release of ethane, when EtPhn was the sole P source (or methane, when MePhn was the sole P source). Therefore, during photoautotrophic growth of *Syn* OS-B′, a significant level of reduced C of the Phn was volatilized and not used for cell growth. *Syn* OS-A, which has neither C–P lyase nor the *phn* transporter genes, continued to grow in the light with MePhn as its sole source of P, but unlike *Syn* OS-B′, failed to accumulate methane in the headspace (Figure 2d). Similar results were observed when EtPhn or AePhn was used as the sole P source; growth was sustained but neither methane nor ethane accumulated in the headspace.

Syn OS-B′ grown in medium containing both Pi and MePhn (Supplementary Figure S1) preferentially used Pi. For cultures supplemented only with 10 μM MePhn, methane evolution began after a 48–72 h lag, which was extended to >96 h when cultures were supplemented with both 10 μM Pi and 10 μM MePhn. This is likely because the Pi has to be exhausted before the cells initiate significant acquisition and use of Phns. The rates of growth of all cultures on the different sources of P were approximately the same.

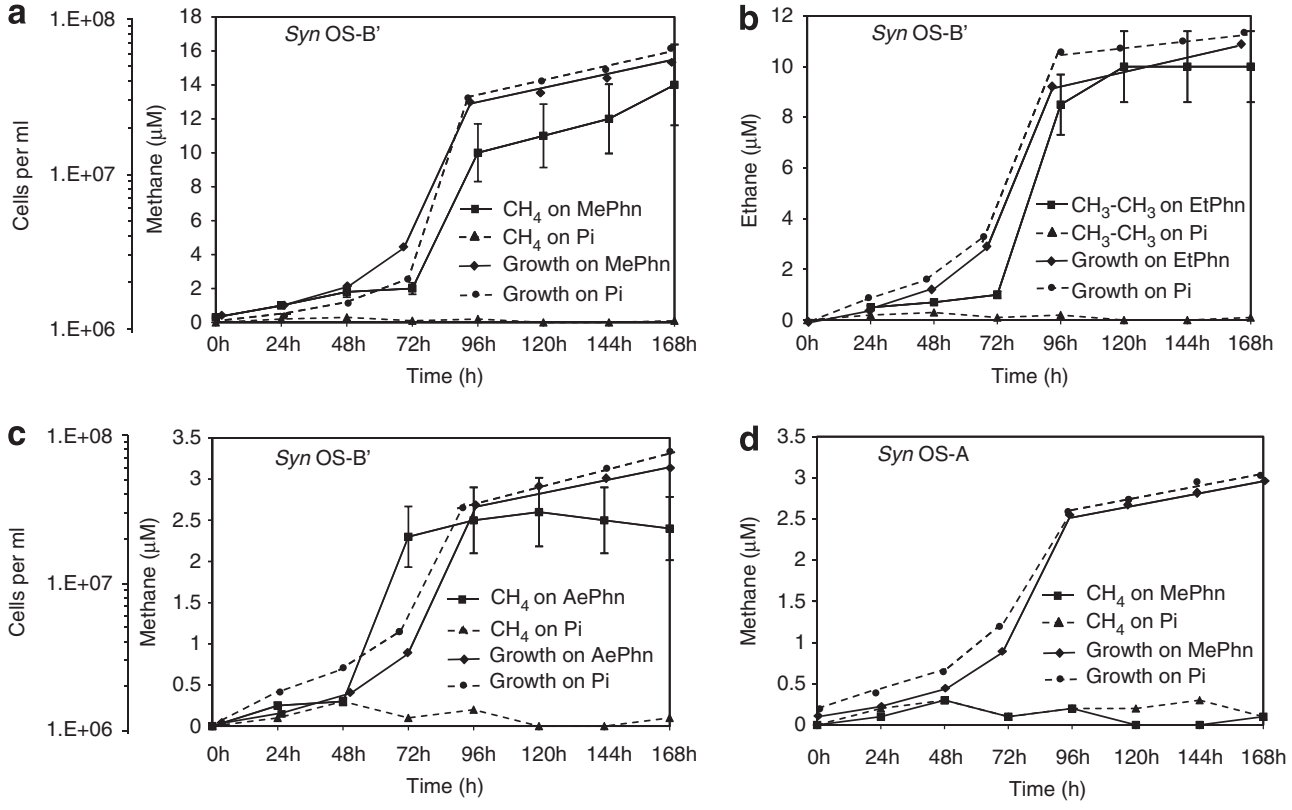

Figure 2 Aerobic growth and accumulation of methane or ethane in Phn-supplemented cultures. Squares and solid lines show accumulation of methane (**a**, **c**) or ethane (**b**) and growth of *Syn* OS-B′ in DH10 (dotted lines) and DH10–P medium supplemented with MePhn (**a**) or EtPhn (**b**) or AePhn (**c**) (solid lines). (**d**) Methane accumulation (squares) and growth (triangles) of *Syn* OS-A in DH10 (Pi, circles and dotted lines) and DH10–P medium supplemented with MePhn (MePhn, solid lines). Cells per ml are shown on a log scale at extreme left. The color reproduction of this figure is available on the html full text version of the manuscript.

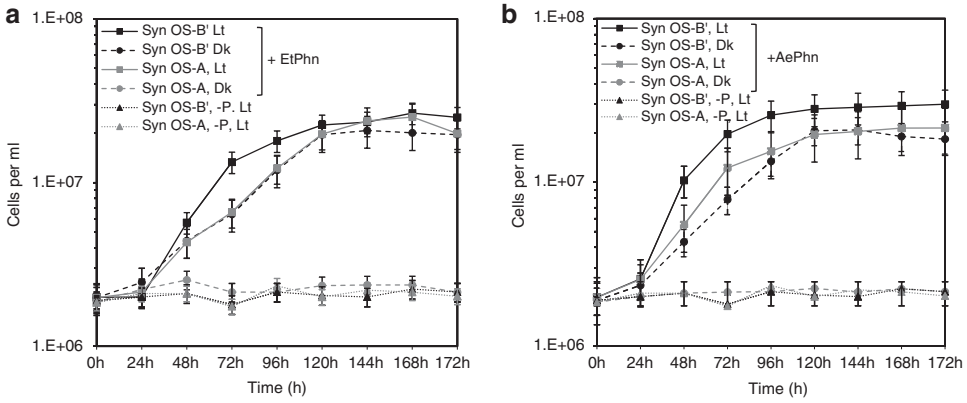

Figure 3 Growth in the light or dark in Phn-supplemented medium. *Syn* OS-A and acclimated *Syn* OS-B′ cells grown in continuous light (75 μmol photon m^{-2} s^{-1}) (gray and black solid lines, respectively) on DH10–P medium supplemented with 0.5 mM of EtPhn (**a**) or AePhn (**b**) or under the same conditions but in continuous dark (dotted lines). As a negative control *Syn* OS-B′ and *Syn* OS-A cells were grown in DH10–P medium in the light (−P, triangles dotted gray lines). The graphs show the means and standard deviations (error bars) of measurements taken from biological triplicates. Cells per ml are shown on a log scale. The color reproduction of this figure is available on the html full text version of the manuscript.

Dark growth

Because *Syn* OS-A and *Syn* OS-B′ have the potential to use different enzyme complexes for Phn assimilation, it was plausible that there would also be differences in the ways in which these organisms use the C backbones of the Phns (Yakovleva *et al.*, 1998). *Syn* OS-B′ can grow in the presence of either EtPhn or AePhn in the light as well as in the dark. In the dark (Figure 3), with EtPhn serving as a sole source of fixed C and P, the cells doubled nearly as rapidly and attained approximately the same cell density as light-grown cells over a period of 172 h (~6–7 doublings). In contrast, acclimated *Syn* OS-B′ cells did not double in the dark on complete medium (P replete, but with no Phn or organic). We conclude that *Syn* OS-B′ can use the C backbone of EtPhn and AePhn as a source of fixed C that fuels cell growth in the dark. As shown in Figure 3b, *Syn* OS-A grew well in the light with EtPhn or AePhn as a sole P source, but neither of these compounds supported the growth of *Syn* OS-A in the dark (Figure 3). Thus, in contrast to *Syn* OS-B′ cells, *Syn* OS-A cells are unable to use Phns as a source of fixed C.

Putative Phnases

These results suggest that *Syn* OS-A and *Syn* OS-B′ may have multiple pathways for Phn acquisition and assimilation. In addition to C–P lyases, bacterial Phnases, such as phosphonoacetaldehyde phosphonohydrolase (*phnA*) and phosphonopyruvate hydrolase, can cleave the C–P bond (Quinn *et al.*, 2007; White and Metcalf, 2007). Phnases belong to the haloacid dehalogenases (HAD) superfamily (Interpro IPR006439), phosphoesterases, ATPases, phosphomutases, dehalogenases and Phnases, which exert their effect on a diverse set of substrates. The HAD domain can be recognized by certain key conserved features such as conserved aspartic acid (Asp) residues that are involved in the phosphotransfer reaction. The Rossman fold that comprises the core in which the phosphotransfer occurs is formed by four loops that contain 'core residues' conserved throughout the HAD superfamily. The conservation of these residues within short motifs and the additions of specific inserts called 'caps' are potential means of identification of specific members of the family (Allen and Dunaway-Mariano, 2004; Lahiri *et al.*, 2004; Burroughs *et al.*, 2006) Despite these general identifying features, Phnases are not very well characterized, so identification based on homology is not definitive.

BLAST searches using the amino-acid sequence encoded by the *phnX* gene (phosphonoacetaldehyde phosphonohydrolase, EC 3.11.1.1) of *Bacillus cereus* AH820 (YP_002450365), which is well characterized at both the catalytic and structural levels, identified at least three putative Phnase-encoding genes on the genomes of *Syn* OS-A and *Syn* OS-B′ (Lahiri *et al.*, 2004). These are CYA_1475 (ortholog is CYB_1669, 74% AAID), CYA_2058 (ortholog is CYB_0748, 90% AAID) and CYA_2485 (ortholog is CYB_2092, 98% AAID). Alignment of PhnX from *B. cereus* and *Pseudomonas aeruginosa* with the putative Phnases of *Syn* OS-A and *Syn* OS- B′ (Supplementary Figure S2) suggests that these may all function as Phnases, but without further biochemical characterization their specificity cannot be easily established.

We estimated the transcript abundance of the putative Phnases under conditions of P starvation, as well as in cultures in which MePhn, EtPhn or AePhn were the sole P source. Transcripts from two putative Phnases (CYA_1475 and CYA_2058) increased in abundance (from ~75- to 225-fold) when cells were grown in medium lacking Pi (2 weeks) and supplemented with either EtPhn or AePhn (MePhn did not elicit accumulation of these

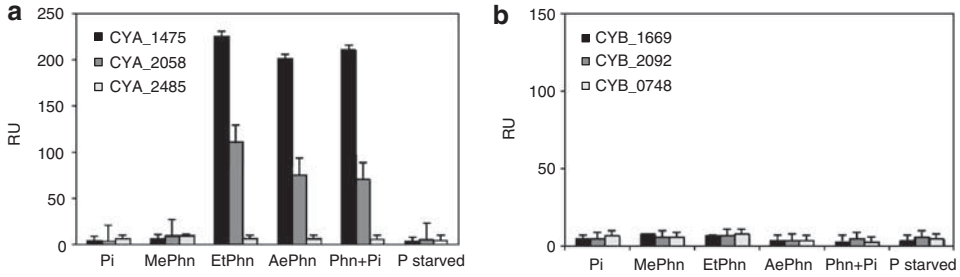

Figure 4 Quantification of transcripts encoding putative Phnases under various growth conditions. Transcripts levels from CYA_1475, CYA_2058 and CYA_2485 of *Syn* OS-A (**a**) and from CYB_1669, CYB_2092 and CYB_0748 of *Syn* OS-B′ (**b**) after growth on DH10 medium (Pi), DH10–P medium (P starved) or DH10–P medium supplemented with MePhn, EtPhn or AePhn and DH10 + P supplemented with MePhn. Samples were taken in exponential phase. qRT-PCR results (relative units, RU) show the means and standard deviations for data from three technical replicates, although similar results were obtained for biological replicates.

transcripts). As expected, the absence of P was not sufficient to elicit accumulation of the putative Phnase transcripts (Figure 4). None of the conditions used elicited a significant change in the level of the CYA_2485 transcript. Furthermore, the CYA_1475 and CYA_2058 transcripts also increased in abundance in cells grown in medium containing Pi and either EtPhn or AePhn, indicating that transcription from the putative Phnase genes is controlled by the presence of the substrate, and is not strongly impacted by the Pi concentration of the medium. Consistent with this result, none of the putative Phnases has a predicted upstream consensus Pho box sequence that has recently been characterized for many cyanobacteria including *Syn* OS-B′ and *Syn* OS-A (Su *et al*., 2007). None of the *Syn* OS-B′ transcripts encoding putative Phnases showed a marked increase in cells grown in medium devoid of P or supplemented with MePhn, EtPhn or AePhn as sole sources of P (Figure 4).

In situ *expression of the key putative Phn utilization genes*

We measured the abundance of transcripts encoding the C–P lyase of *Syn* OS-B′ and the Phnases of *Syn* OS-A in mat samples collected from Octopus Spring at different times of the diel cycle on 28–29 July 2007. qRT-PCR with specific primers was used to evaluate *phn*C (gene in C–P lyase operon of *Syn* OS-B′) and the putative Phnase (CYA_1475 and CYA_2058) transcript levels of *Syn* OS-A. The *phn*C transcript was highest in the evening, when irradiance levels were low and the mat was anoxic (Figure 5), with a subsequent decline in the transcript level over the course of the night. This transcript may be controlled by oxic conditions and/or the circadian program. The levels of transcripts for the *Syn* OS-A putative Phnases are highest at mid afternoon when the mat is still oxic, and then decline. Expression of putative Phnases of *Syn* OS-B′ was significantly lower than for the putative orthologs in *Syn* OS-A, and there was no clear trend in the pattern of expression (Supplementary Table S3).

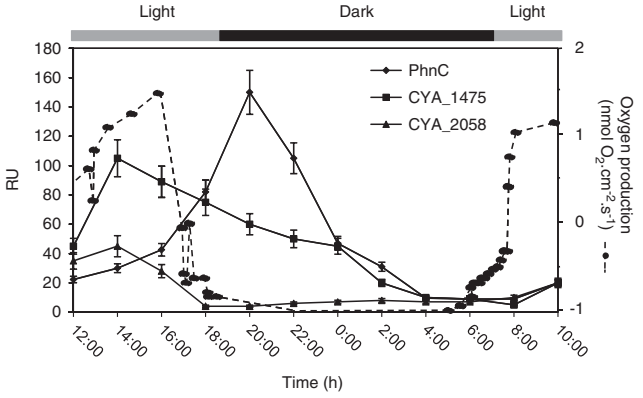

Figure 5 *In situ* quantification of transcripts from putative Phnases (CYA_1475, CYA_2058) and PhnC over the diel cycle. Oxygen production is shown as the dotted curve; oxic (light) and anoxic (dark) state of the mat indicated by gray and black bars, respectively. Transcript levels (relative units, RU) of genes encoding the Phn transporter (PhnC, CYB_0159, diamonds) and the putative Phnases from *Syn* OS-A (CYA_1475, squares, CYA_2058, triangles) are shown. The color reproduction of this figure is available on the html full text version of the manuscript.

Discussion

Microorganisms in the environment are capable of synthesizing, taking up and using Phns (Kononova and Nesmeyanova, 2002; Quinn *et al*., 2007; White and Metcalf, 2007; Metcalf and van der Donk, 2009; Singh, 2009). Pathways for Phn biosynthesis are less well-studied than those for its degradation (Quinn *et al*., 2007) and analysis of genomic and metagenome data available from the hot springs (Bhaya *et al*., 2007) did not provide compelling evidence that Phn biosynthetic pathways are present in the microbial mat community. However, we did demonstrate that *Syn* OS-B′ can grow well in medium in which the only source of P is MePhn, EtPhn or AePhn, contingent on an acclimation period of approximately 3 weeks in the presence of Phn compounds (Adams *et al*., 2008). Over the initial period of acclimation, the presence of Phn in the medium suppressed the growth of the cells on Pi,

suggesting that Phns may function as an antimetabolites and block certain cellular activities (Kononova and Nesmeyanova, 2002).

Syn OS-B′ can use MePhn or EtPhn as a sole P source in the light, and as a consequence cells release either methane or ethane. These results suggest that in the light, *Syn* OS-B′ cells take up Phns, which is then further metabolized by the cytoplasmic C–P lyase. Under these conditions, released Pi is assimilated whereas some of the reduced carbon moiety of the Phn is released into the atmosphere as methane or ethane. However, in the dark, the reduced C moiety of the Phn is integrated into the metabolic circuitry of the cell, may be through conversion to acetyl CoA. In other systems that have been studied, biodegradation appears to involve ribosylated organophosphonate intermediates (Avila *et al*., 1991; Hove-Jensen *et al*., 2010) and possibly result in the generation of more than one reduced C species. Transcripts encoding Phn transporter/C–P lyase subunits increase when *Syn* OS-B′ cells are starved for P (in the absence or presence of MePhn, EtPhn or AePhn), whereas the Phnase transcripts are not. Therefore, it is most likely that the Phn transporter/C–P lyase system is important for Phn use in *Syn* OS-B′ under the conditions used in this study (although we cannot eliminate the possibility that some Phn use is a consequence of Phnases). The finding that little, if any, of the organic C is released as a volatile compound in the dark, and that cells grow in the dark when the only reduced C is in the form of Phn, strongly indicates that *Syn* OS-B′ can exploit the energetic component of Phn at a time when it is limited in its ability to generate chemical bond energy through either photosynthesis or respiratory metabolism. Indeed, the Phn transport/C–P lyase system would allow *Syn* OS-B′ cells to transport the entire Phn molecule into the cytoplasm, where the C–P bond would be cleaved. The C backbones released could be integrated into assimilation pathways through the activities of enzymes such as acetyl-CoA synthetase (CYB_0240, CYA_0472) and ATP citrate lyase (CYB_1908, CYA_1739).

In contrast to *Syn* OS-B′, *Syn* OS-A maintained in medium containing MePhn, EtPhn or AePhn did not emit methane or ethane either in the light or dark and was unable to use any of these Phn compounds as a sole C source for growth in the dark. Apparently, *Syn* OS-A can access the P moiety of Phn, but cannot metabolize the reduced C moiety of the molecule. *Syn* OS-A appears to use various Phnases and two genes encoding putative Phnases are induced during exposure of *Syn* OS-A to specific Phn substrates. In *Salmonella* sp. Phnase is induced under P starvation (Jiang *et al*., 1995) but in *Rhizobium huakuii* PMY1, Phnase are not under control of the Pho regulon. The ability of *Syn* OS-A to induce specific Phnases, in the presence of P, might indicate that Phns are available in these environments. However, information regarding the source, levels and use of P sources, including Phns, in many environments, including hot springs is limited and suffers from technical challenges (Stauffer and Thompson, 1978; Stauffer, 1982; Papke *et al*., 2003).

Some Phnases may be membrane associated potentially accessing the extracellular environment (Mendz *et al*., 2005), as a consequence, Pi may be generated in the extracellular space and then rapidly taken up and assimilated by the cells. If so, *Syn* OS-A might be unable to use EtPhn or AePhn as a sole source of C because it cannot take up the extracellular C compound released during Phn catalysis. Phnase transcripts from CYA_1475 and CYA_2058 accumulate in the presence of EtPhn and AePhn, but not MePhn, and yet *Syn* OS-A can use all of these compounds as P sources. These results suggest that other as yet unidentified Phnases may be involved in the use of MePhn by *Syn* OS-A, and that substrate specificity may be associated with each of the putative Phnases (Kononova and Nesmeyanova, 2002; White and Metcalf, 2007). There may be other novel mechanisms by which Phns are degraded by microorganisms (Fox and Mendz, 2006; Quinn *et al*., 2007).

Recently, Karl *et al*. (2008) have shown that microbial Phn degradation results in methane generation in aerobic marine environments, providing evidence for 'unconventional' methanogenesis. This bacterial catabolism of Phns might explain why oceanic methane concentrations vastly exceed the levels predicted by basic biological and chemical principles (Ingall, 2008). The ability of *Trichodesmium* sp. to metabolize as well as produce Phn compounds may explain why this cyanobacterium is so successful in low-P environments (Dyhrman and Haley, 2006; Dyhrman *et al*., 2007, 2009). Marine unicellular cyanobacteria contain putative Phn transporters and Phnases (Tetu *et al*., 2009). Marine bacteria including *Vibro* sp. and *Planctomyces* sp. have the ability to grow on Phns (Dyhrman *et al*., 2007). Moreover, the widespread distribution of the *phnA* gene, which encodes a phosphonoacetate hydrolase, underscores the potential importance of phosphonates in marine ecosystems, including corals (Gilbert *et al*., 2009; Martinez *et al*., 2009; Thomas *et al*., 2010).

Methane production in low-sulfate, hot-spring algal–bacterial mats has been ascribed to methanogenic bacteria as well as eukaryotic organisms (Ward, 1978). Here we show that the Phn transporter/C–P lyase genes and putative Phnase genes are expressed *in situ*, so cyanobacteria could produce methane as a by-product of Phn metabolism (levels of Phns in these environments have not been measured). Transcript profiles suggest that the Phn transporter/C–P lyase genes are expressed when the mat is anoxic, suggesting that the level of the Phn transporter/C–P lyase is higher at night. In contrast, Phnase genes are expressed in mid afternoon when the cells are photosynthesizing and are not likely to require additional fixed C. Hence, Phn cleavage, possibly in the extracellular environment during the

day, would allow cells to capture the P in the molecule whereas the fixed C moiety would be released into the environment where it could be exploited by heterotrophs in the mat. These results highlight the ability of terrestrial cyanobacteria to use multiple systems to acquire and metabolize Phns from the environment, but investigations to elucidate the precise role of these specific activities in shaping population dynamics and micro-niche habitation are only just beginning.

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References

Adams MM, Gomez-Garcia MR, Grossman AR, Bhaya D. (2008). Phosphorus deprivation responses and phosphonate utilization in a thermophilic *Synechococcus* sp. from microbial mats. *J Bacteriol* **190**: 8171–8184.

Allen KN, Dunaway-Mariano D. (2004). Phosphoryl group transfer: evolution of a catalytic scaffold. *Trends Biochem Sci* **29**: 495–503.

Allewalt JP, Bateson MM, Revsbech NP, Slack K, Ward DM. (2006). Effect of temperature and light on growth of and photosynthesis by *Synechococcus* isolates typical of those predominating in the Octopus Spring microbial mat community of Yellowstone National Park. *Appl Environ Microbiol* **72**: 544–550.

Avila LZ, Draths KM, Frost JW. (1991). Metabolites associated with organophosphonate C–P bond cleavage: chemical synthesis and microbial degradation of [^{32}P]-ethylphosphonic acid. *Bioorgan Med Chem* **1**: 51–54.

Benitez-Nelson CR, O'Neill L, Kolowith LC, Pellecia P, Thunell R. (2004). Phosphonates and particulate organic phosphorus cycling in an anoxic marine basin. *Limnol Oceanogr* **49**: 1593–1604.

Bhaya D, Grossman AR, Steunou AS, Khuri N, Cohan FM, Hamamura N *et al.* (2007). Population level functional diversity in a microbial community revealed by comparative genomic and metagenomic analyses. *ISME J* **1**: 703–713.

Burroughs AM, Allen KN, Dunaway-Mariano D, Aravind L. (2006). Evolutionary genomics of the HAD superfamily: understanding the structural adaptations and catalytic diversity in a superfamily of phosphoesterases and allied enzymes. *J Mol Biol* **361**: 1003–1034.

Clark LL, Ingall ED, Benner R. (1998). Marine phosphorus is selectively remineralized. *Nature* **393**: 426.

Cook AM, Daughton CG, Alexander M. (1978). Phosphonate utilization by bacteria. *J Bacteriol* **133**: 85–90.

Dyhrman ST, Ammerman JW, Van Mooy BAS. (2007). Microbes and the marine phosphorus cycle. *Oceanography* **20**: 110–116.

Dyhrman ST, Benitez-Nelson CR, Orchard ED, Haley ST, Pellechia PJ. (2009). A microbial source of phosphonates in oligotrophic marine systems. *Nat Geosci* **2**: 696–699.

Dyhrman ST, Chappell PD, Haley ST, Moffett JW, Orchard ED, Waterbury JB *et al.* (2006). Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. *Nature* **439**: 68–71.

Dyhrman ST, Haley ST. (2006). Phosphorus scavenging in the unicellular marine diazotroph *Crocosphaera watsonii*. *Appl Environ Microbiol* **72**: 1452–1458.

Elser JJ, Matthew ES, Bracken E, Cleland E, Daniel S, Gruner W *et al.* (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol Lett* **10**: 1135–1142.

Falkowski PG, Fenchel T, Delong EF. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science* **320**: 1034–1039.

Forlani G, Pavan M, Gramek M, Kafarski P, Lipok J. (2008). Biochemical bases for a widespread tolerance of cyanobacteria to the phosphonate herbicide glyphosate. *Plant Cell Physiol* **49**: 443–456.

Fox EA, Mendz GL. (2006). Phosphonate degradation in microorganisms. *Enzyme Microb Technol* **40**: 145–150.

Gilbert JA, Thomas S, Cooley NA, Kulakova A, Field D, Booth T *et al.* (2009). Potential for phosphonoacetate utilization by marine bacteria in temperate coastal waters. *Environ Microbiol* **11**: 111–125.

Hildebrand RL, Henderson TG. (1983). Phosphonic acids in nature. In Hildebrand, RL (ed.). *The Role of Phosphonates in Living Systems*. CRC Press: Boca Raton, FL.

Hove-Jensen B, Rosenkrantz TJ, Zechel DL, Willemoes M. (2010). Accumulation of intermediates of the carbon–phosphorus lyase pathway for phosphonate degradation in *phn* mutants of *Escherichia coli*. *J Bacteriol* **192**: 370–374.

Ilikchyan IN, McKay RM, Zehr JP, Dyhrman ST, Bullerjahn GS. (2009). Detection and expression of the phosphonate transporter gene *phn*D in marine and freshwater picocyanobacteria. *Environ Microbiol* **11**: 1314–1324.

Ingall ED. (2008). Making methane. *Nat Geoscience* **1**: 419–420.

Iqbal S, Parker G, Davidson H, Moslehi-Rahmani E, Robson RL. (2004). Reversible phase variation in the *phnE* gene, which is required for phosphonate metabolism in *Escherichia coli* K-12. *J Bacteriol* **186**: 6118–6123.

Jiang W, Metcalf WW, Lee KS, Wanner BL. (1995). Molecular cloning, mapping, and regulation of Pho regulon genes for phosphonate breakdown by the phosphonatase pathway of *Salmonella typhimurium* LT2. *J Bacteriol* **177**: 6411–6421.

Karl DM, Beversdorf L, Björkman KM, Church MJ, Martinez A, Delong EF. (2008). Aerobic production of methane in the sea. *Nat Geosci* **1**: 473–478.

Kilian O, Steunou AS, Fazeli F, Bailey S, Bhaya D, Grossman AR. (2007). Responses of a thermophilic *Synechococcus* isolate from the microbial mat of Octopus Spring to light. *Appl Environ Microbiol* **73**: 4268–4278.

Kolowith LC, Ingall ED, Benner R. (2001). Composition and cycling of marine organic phosphorus. *Limnol Oceanogr* **46**: 309–320.

Kononova SV, Nesmeyanova MA. (2002). Phosphonates and their degradation by microorganisms. *Biochemistry (Mosc)* **67**: 184–195, 184–195.

Lahiri SD, Zhang G, Dai J, Dunaway-Mariano D, Allen KN. (2004). Analysis of the substrate specificity loop of the HAD superfamily cap domain. *Biochemistry* **43**: 2812–2820.

Martinez A, Tyson GW, Delong EF. (2009). Widespread known and novel phosphonate utilization pathways in marine bacteria revealed by functional screening and metagenomic analyses. *Environ Microbiol* **12**: 222–238.

Mendz GL, Megraud F, Korolik V. (2005). Phosphonate catabolism by *Campylobacter* spp. *Arch Microbiol* **183**: 113–120.

Metcalf WW, van der Donk WA. (2009). Biosynthesis of phosphonic and phosphinic acid natural products. *Annu Rev Biochem* **78**: 65–94.

Mills MM, Ridame C, Davey M, La Roche J, Geider RJ. (2004). Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. *Nature* **429**: 292–294.

Nowack B. (2003). Environmental chemistry of phosphonates. *Water Res* **37**: 2533–2546.

Orchard ED, Webb EA, Dyhrman ST. (2009). Molecular analysis of the phosphorus starvation response in *Trichodesmium* spp. *Environ Microbiol* **11**: 2400–2411.

Papke RT, Ramsing NB, Bateson MM, Ward DM. (2003). Geographical isolation in hot spring cyanobacteria. *Environ Microbiol* **5**: 650–659.

Quinn JP, Kulakova AN, Cooley NA, McGrath JW. (2007). New ways to break an old bond: the bacterial carbon–phosphorus hydrolases and their role in biogeochemical phosphorus cycling. *Environ Microbiol* **9**: 2392–2400.

Rozen S, Skaletsky H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**: 365–386.

Sanudo-Wilhelmy SA. (2006). Oceanography: a phosphate alternative. *Nature* **439**: 25–26.

Singh BK. (2009). Organophosphorus-degrading bacteria: ecology and industrial applications. *Nat Microbiol Rev* **7**: 156–164.

Stauffer RE. (1982). Fluorapatite and fluorite solubility controls on geothermal waters in Yellowstone National Park. *Geochemica Cosmochemica Acta* **46**: 465–474.

Stauffer RE, Thompson JM. (1978). Phosphorous in hydrothermal waters of Yellowstone National Park, Wyoming. *J Res US Geol Surv* **6**: 755–763.

Steunou AS, Bhaya D, Bateson MM, Melendrez MC, Ward DM, Brecht E *et al.* (2006). *In situ* analysis of nitrogen fixation and metabolic switching in unicellular thermophilic cyanobacteria inhabiting hot spring microbial mats. *Proc Natl Acad Sci USA* **103**: 2398–2403.

Steunou AS, Jensen SI, Brecht E, Becraft ED, Bateson MM, Kilian O *et al.* (2008). Regulation of *nif* gene expression and the energetics of N_2 fixation over the diel cycle in a hot spring microbial mat. *ISME J* **2**: 364–378.

Su Z, Olman V, Xu Y. (2007). Computational prediction of Pho regulons in cyanobacteria. *BMC Genomics* **8**: 156–167.

Ternan NG, Mc Grath JW, Mc Mullan G, Quinn JP. (1998). Organophosphonates: occurrence, synthesis and biodegradation by microorganisms. *World J Microbiol Biotech* **14**: 635–647.

Tetu SG, Brahamsha B, Johnson DA, Tai V, Phillippy K, Palenik B *et al.* (2009). Microarray analysis of phosphate regulation in the marine cyanobacterium *Synechococcus* sp. WH8102. *ISME J* **3**: 835–849.

Thomas S, Burdett H, Temperton B, Wick R, Snelling D, McGrath JW *et al.* (2010). Evidence for phosphonate usage in the coral holobiont. *ISME J* **4**: 459–461.

Torriani-Gorini A, Yagil E, Simon S. (1994). *Phosphate in Microorganisms: Cellular and Molecular Biology*. ASM Press: Washington, DC.

Van Mooy BA, Fredricks HF, Pedler BE, Dyhrman ST, Karl DM, Koblízek M *et al.* (2009). Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature* **458**: 69–72.

Ward DM. (1978). Thermophilic methanogenesis in a hot-spring algal–bacterial mat (71 to 30 degrees C). *Appl Environ Microbiol* **35**: 1019–1026.

Whelan JA, Russell NB, Whelan MA. (2003). A method for the absolute quantification of cDNA using real-time PCR. *J Immunol Methods* **278**: 261–269.

White AK, Metcalf WW. (2007). Microbial metabolism of reduced phosphorus compounds. *Annu Rev Microbiol* **61**: 379–400.

Wu J, Sunda W, Boyle EA, Karl DM. (2000). Phosphate depletion in the western North Atlantic Ocean. *Science* **289**: 759–762.

Yakovleva GM, Kim SK, Wanner BL. (1998). Phosphate-independent expression of the carbon–phosphorus lyase activity of *Escherichia coli*. *Appl Microbiol Biotechnol* **49**: 573–578.

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