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Ecotypic variation in phosphorus-acquisition mechanisms within marine picocyanobacteria

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ABSTRACT: *Prochlorococcus* and *Synechococcus* are major prokaryotic primary producers in the oligotrophic oceans that may be affected by the climate-related increases in nitrogen fixation and subsequent phosphorus (P) limitation in some parts of the oceans. Evidence that *Prochlorococcus* populations in the North Pacific subtropical gyre (NPSG) have increased over the past decades, possibly due to having a competitive advantage under conditions of P limitation, suggests aspects of their P physiology that are important for dictating their *in situ* success. Here, we compared the physiology of P acquisition and response to P stress (indicated by alkaline phosphatase activity, APA) among isolates of *Prochlorococcus* and *Synechococcus* representing different genotypic clades within the marine picophytoplankton lineage (sensu Urbach et al. 1998: J Mol Evol 46:188–201). The 2 *Synechococcus* isolates examined (WH 8102 and WH 7803) can utilize a wide variety of organic P sources. Of the 3 *Prochlorococcus* isolates examined, only the HLI genotype, MED4, is capable of growth on a variety of organic P sources. Under conditions of P starvation the 2 *Synechococcus* strains and *Prochlorococcus* MED4 exhibit significant increases in APA, above their measurable constitutive activities. The genomes of the *Synechococcus* strains and *Prochlorococcus* MED4 indicate the presence of many P-uptake and -regulatory genes required under conditions of P stress, including the *phoA* gene that encodes for an alkaline phosphatase enzyme. The other isolates of *Prochlorococcus*, HLII MIT 9312 and LLIV MIT 9313, have distinctly different P-stress responses. MIT 9312, which contains the same P-uptake and -regulatory genes as MED4, except for *psip1* and *ptrA*, has no constitutive APA, but does exhibit measurable, albeit very low, activity when P starved. MIT 9313, which lacks *phoA* and a functional phosphate-sensing histidine kinase gene, *phoR*, exhibits low constitutive activity that decreases when the cells become P-starved. These results show variability in P utilization and in P-stress response between the 2 genera of marine unicellular cyanobacteria, and marked differences among *Prochlorococcus* genotypes, implying that only certain eco/genotypes of these marine cyanobacteria will have an ecological advantage under conditions of P limitation in the oceans. This further points to the critical need to continue developing genotypic- or cell-specific tools to assess the response of the picophytoplankton community to large-scale changes in nutrient conditions in the oceans.

KEY WORDS: Marine cyanobacteria · *Prochlorococcus* · *Synechococcus* · Phosphorus starvation · Alkaline phosphatase activity · APA

INTRODUCTION

In the world's oligotrophic oceanic regions, nitrogen (N) and iron are traditionally considered the nutrients that limit primary production (Graziano et al. 1996, Mills et al. 2004). However, at certain times of the year, phosphorus (P) may actually limit primary production. The Mediterranean Sea appears to be P-limited during summer-stratified periods (Thingstad & Rassoulzadegan 1995). In the North Atlantic, a seasonal P limitation (based on measurements of concentrations and ratios of N and P and alkaline phosphatase activity, APA) has been observed following increased periods of N_2 fixation (Michaels et al. 1996, Wu et al. 2000, Cavender-

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Bares et al. 2001, Ammerman et al. 2003). The most dramatic example of P limitation has been documented in the North Pacific subtropical gyre (NPSG), where it appears to have undergone a climate-related shift from an N- to a P-limited ecosystem over the past several decades, due to increases in N_2 fixation (Karl et al. 1997). Correlated with increased N_2 fixation are shifts in the total dissolved nutrients to greater concentrations of organic P compounds in the euphotic water column of the Sargasso Sea and NPSG (Bjorkman et al. 2000). This shift to P limitation may also be the cause of an observed 'domain shift' toward an ecosystem dominated by prokaryotic organisms (Karl et al. 2001). Thus, these short- and long-term shifts in P limitation are affecting rates of gross primary production and export, C:N:P stoichiometry of dissolved and particulate matter, and community structure.

The unicellular marine cyanobacterial genera, *Prochlorococcus* and *Synechococcus*, dominate the photosynthetic biomass and are responsible for a significant portion of primary production in oligotrophic ocean ecosystems (Partensky et al. 1999). However, whether these primary producers become P-limited is not clear. There is some evidence in the Red Sea (Li et al. 1998, Fuller et al. 2005) and the Mediterranean Sea (Vaulot et al. 1996) that *Synechococcus* populations may be P-limited, whereas *Prochlorococcus* populations do not appear to be (Vaulot & Partensky 1992). On the other hand, it has recently been reported that *Synechococcus* predominate in the Mediterranean Sea, possibly due to their high affinity for inorganic P (P_i) and significantly higher maximum uptake rates relative to other plankton (Moutin et al. 2002). In the NPSG *Prochlorococcus* populations appear to have increased over the last several decades, possibly in response to the shift towards conditions of P limitation (Karl et al. 2001). These results on natural populations of *Prochlorococcus* and *Synechococcus* may differ if different eco/genotypes[1] of either of these genera were dominating at the time of the studies. Regardless, both genera appear to be impacted in some way by conditions of P limitation.

Isolates of both *Prochlorococcus* and *Synechococcus* have been categorized based on their phylogenetic relationships, which in many cases can be correlated to differences in their ecology and/or physiology. There are 2 physiologically and genetically distinct groups of *Prochlorococcus* isolates: high-light-adapted isolates (HL ecotypes) and low-light-adapted isolates (LL ecotypes), which differ in their chlorophyll *b*/*a* ratio, photosynthetic capabilities, copper sensitivity, nitrite utilization, and depth distributions (Moore et al. 1998, Moore & Chisholm 1999, Rocap et al. 1999, West & Scanlan 1999, West et al. 2001, Mann et al. 2002). The HL isolates can be further subdivided into HLI and HLII genotypes, the ecophysiological significance of which is not yet evident. *Synechococcus* isolates have been split into as many as 10 clades, only 2 of which have a distinctive physiology associated with them (Toledo & Palenik 1997, Urbach et al. 1998, Toledo et al. 1999, Rocap et al. 2002, Fuller et al. 2003). Up to this point, phosphorus physiology as a potential determinant of these different eco/genotypes has not been examined.

Extensive studies of marine A *Synechococcus* Strain WH 7803 (Clade V) indicate that this strain can use a wide range of organic P sources for growth and that P limitation results in high P_i-uptake rates and production of a high-affinity P_i-binding protein (Scanlan et al. 1993, Donald et al. 1997). Examination of the P physiology of *Prochlorococcus* has not been as extensive, due to the difficulty in studying the physiological status of natural populations and to the difficulty in obtaining isolates of *Prochlorococcus* that are free of heterotrophic contaminants, i.e. axenic. Cell-cycle studies of *Prochlorococcus* indicate that their response to P starvation is strikingly different from that of *Synechococcus* (Parpais et al. 1996). Studies on the axenic *Prochlorococcus* sp. PCC 9511 indicated that, like *Synechococcus* WH 7803, this strain of *Prochlorococcus* can utilize a wide variety of organic P sources for growth (Rippka et al. 2000). Recent work on the elemental composition of *Prochlorococcus* and *Synechococcus* isolates under both P-replete and P-limited culture conditions indicate that their C:N:P ratios are higher than the Redfield ratio, suggesting relatively low P requirements and potential competitive advantage over heterotrophic bacteria and larger phytoplankton in oligotrophic oceans (Bertilsson et al. 2003, Heldal et al. 2003). A recent study by Fuller et al. (2005) indicates that natural populations of *Prochlorococcus* (dominated by the HLII genotype) in the Red Sea appear unaffected by P stress, whereas *Synechococcus* populations may have declined due to P limitation.

The recent availability of the complete genome sequences for motile Clade III *Synechococcus* WH 8102 (Palenik et al. 2003) and 3 *Prochlorococcus* strains (Dufresne et al. 2003, Rocap et al. 2003) has provided insight into the genetic basis relating to P ecophysiology. *Synechococcus* WH 8102 and HLI *Prochloro-*

[1]Different types of marine *Synechococcus* and *Prochlorococcus* have been referred to as ecotypes, genotypes, or pigment types by different researchers. In this paper, the term 'ecotype' will refer to phylogenetic clades of *Prochlorococcus* that have a characteristic ecophysiology, and 'genotype' will be used when referring to phylogenetic clades of either *Synechococcus* or *Prochlorococcus* that do not yet have an associated ecophysiology

coccus MED4 possess most of the genes required for P acquisition and regulation, whereas the other 2 *Prochlorococcus* genomes, LL ecotypes MIT 9313 and SS120, lack several of these genes, indicating clear diversity among the *Prochlorococcus* ecotypes (Scanlan & West 2002, Mary & Vaulot 2003). This genomic information and the experiments to date are intriguing, but do not provide a complete or clear picture of how these ecologically significant cyanobacteria deal with P limitation, especially with respect to the diversity among *Prochlorococcus* and *Synechococcus* eco/genotypes. In order to gain a better understanding of the P physiology and to further our ecological understanding of these 2 cyanobacterial genera, we undertook a comparative assessment of the ability to utilize different P sources and the alkaline phosphatase response to P starvation for a variety of axenic strains from both genera.

MATERIALS AND METHODS

Cultures and stock growth conditions. Three axenic *Prochlorococcus* isolates representing 3 distinct phylogenetic lineages (MED4 [Clade HLI], MIT 9312 [Clade HLII], and MIT 9313 [Clade LLIV]) and 5 axenic *Synechococcus* isolates representing 4 distinct genotypes (WH 7803 [Clade V], WH 8102 and WH 8103 [Clade III], WH 8012 [Clade I], and MIT S9220 [Clade VII]) were used. All *Prochlorococcus* cultures and *Synechococcus* MIT S9220 were obtained by courtesy of S. W. Chisholm (Massachusetts Institute of Technology). *Synechococcus* Strains WH 7803, WH 8103, and WH 8012 were obtained from J. Waterbury (Woods Hole Oceanographic Institution), and WH 8102 was obtained from B. Brahamsha (Scripps Institution of Oceanography).

Prochlorococcus MIT 9313 and *Synechococcus* MIT S9220 were rendered axenic by flow cytometric sorting on a DakoCytomation MoFlo following methods outlined in Sieracki et al. (2004). Sample lines were sterilized with bleach, then rinsed with sterile MilliQ H_2O before sorting. Sterile, filtered seawater was used for the sheath. In order to ensure high purity, single-sort mode with a sort envelope of 0.5 drops was used. Two cells were sorted per tube of sterile, standard Sargasso Sea water-based media (Moore et al. 2002) from a sort region positioned at the center of the flow cytometric signature specific for the culture used. Sorted cultures were followed for growth and contamination for several months before an axenic culture was verified and reliably transferable.

Stock cultures of *Prochlorococcus* and *Synechococcus* WH 8103, WH 8012, and MIT S9220 were maintained in a standard Sargasso Sea water-based medium (Moore et al. 2002) at a constant temperature of 24°C and ~60 µmol Q m^{-2} s^{-1} on a 14 h light:10 h dark cycle. Additional stock cultures of *Synechococcus* WH 8102 and WH 7803 were maintained in artificial seawater medium (ASW; Rippka et al. 2000), at a constant temperature of 24°C and constant light of 15 µmol Q m^{-2} s^{-1} for WH 7803 and 30 µmol Q m^{-2} s^{-1} for WH 8102. All stock and experimental cultures were monitored using Marine Purity Broth (Bertilsson et al. 2003) and/or epifluorescence microscopic visualization of cultures stained with DAPI or SYBR Green (Molecular Probes) at various times to verify that they remained axenic.

Growth-capability experiments. To test the ability to grow on a variety of environmentally relevant P sources (Karl & Yanagi 1997, Kolowith et al. 2001), axenic stock cultures of *Prochlorococcus* and *Synechococcus* were transferred to duplicate tubes of Sargasso Sea water-based medium containing the same trace metal mix as the stock media, 500 µM NH_4Cl, and 10 µM of P (tripolyphosphate [PPP_i], β-glycerophosphate [GYP], glucose-6-phosphate [G6P], adenosine triphosphate [ATP], 3′, 5′-cyclic adenosine monophosphate [cAMP], or 2-aminoethylphosphonic acid [2AEP]). A control culture without added P (noP) was always grown in parallel when testing growth capability. Growth was measured by following daily changes in autofluorescence using a Turner TD-700 fluorometer or cell concentration as determined by flow cytometry (Becton-Dickinson FACS-Calibur). Additional experiments were carried out with *Synechococcus* WH 7803 and WH 8102 grown in duplicate in ASW medium in which PO_4 was replaced by 10 µM G6P, ATP, or cAMP. Growth on specific P sources was verified through serial transfers on the same P source, whereas lack of growth on a particular P source was verified by testing several times.

P-stress response experiments. To assess the P-stress response, alkaline phosphatase-like activity (APA) was measured over the growth cycle for selected isolates of *Prochlorococcus* (MED4, MIT 9312, and MIT 9313) and *Synechococcus* (WH 8102 and WH 7803). These particular experiments were carried out in different laboratories, and thus different methods for growth and APA were used, as outlined below. Despite the different methods, however, the trends in the physiological results between the 2 genera of cyanobacteria are comparable. For *Prochlorococcus* experiments, cells were grown in 250 or 500 ml batch cultures containing Sargasso Sea water-based media with the trace metal mix, 250 µM NH_4Cl and 1 µM of NaH_2PO_4 (N:P = 250:1). Throughout exponential growth and into the P-starved stationary phase, cell concentration was determined by flow cytometry (Becton-Dickinson FACS-Calibur), soluble reactive phosphorus (SRP)

concentration was determined spectrophotometrically (Cary 50Bio UV/Vis-spectrophotometer; Murphy & Riley 1962), and alkaline phosphatase activity was measured fluorometrically (SPEX FluoroMax-2) using the fluorogenic compound methylumbelliferyl phosphate (MUFP). Briefly, MUFP (200 µM final concentration) was added directly to subsamples of the cultures, and fluorescence emission of MUF product was measured at 442 nm as a function of time. Standard curves of MUF (Sigma, M-1508) were generated to convert the fluorescence emission data to PO_4 concentrations, assuming equimolar generation of MUF and PO_4 by APase. Rates were normalized to cell number (units of amol P h^{-1} $cell^{-1}$).

Synechococcus strains were transferred to 100 ml batch cultures of ASW containing 10 µM KH_2PO_4 for 2 successive transfers before analysis. APA was measured throughout the growth curve with the para-nitrophenyl phosphate (p-NPP) assay (Bessey et al. 1946) adapted for use in a microplate reader. Briefly, an aliquot of cells (160 µl) was incubated with a solution of p-NPP or bis-p-NPP (18 mM, Sigma, 104 phosphatase substrate or bis-p-NPP, Acros Organics), in 1 M Tris-HCl (pH 8.0) to give a final concentration of 3.6 mM p-NPP and 0.2 M Tris (pH 8.0) in 200 µl (concentrations of up to 40 mM p-NPP resulted in the same APA rates, indicating that 3.6 mM p-NPP was saturating). The formation of product (pNP) was measured in kinetic mode over a period of 5 h by the change in absorbance at 405 nm on a Thermo Labsystems iEMS plate reader. APA was measured in triplicate from at least 3 independent experiments and calculated from the linear portion of the curve by subtracting the slope of control wells containing either no substrate or no cells. The formation of colored substrate was linear over the assay period, apart from the first 15 min. Absorbance units were converted to pNP concentration with the extinction coefficient 1.78×10^4 M^{-1} cm^{-1}, at pH 8.0, and a light path length of 0.32 cm per assay well, and then equated to PO_4 following the same assumption as with the MUF conversion. Cell counts were determined by flow cytometry and used for normalizing APA values (units of amol PO_4 h^{-1} $cell^{-1}$).

Genomic analysis. The presence (or absence) of genes involved in uptake, utilization, and regulation of P_i and organic P were assessed from publicly available genome sequences of *Prochlorococcus* MED4 (GenBank Accession Number NC_005072), MIT 9313 (NC_005071), SS120 (NC_005040), and *Synechococcus* WH 8102 (NC_005070). The closed genome sequences of MIT 9312 (M. Coleman, C. Steglich, and S. W. Chisholm pers. comm.) and *Synechococcus* WH 7803 (F. Partensky pers. comm.) were made available prior to public release specifically for use in this publication. Genome annotations available in GenBank and draft annotations of WH 7803 and MIT 9312 were used to guide gene assignments. The finished MIT 9312 genome is now available at DOE's Joint Genome Institute's Microbial Genomics website (http://genome.jgi-psf.org/microbial/). The presence or absence of genes in each genome was determined by reciprocal BLAST searches on the basis that orthologous pairs demonstrated >50% identity over 80% of each protein sequence, unless otherwise stated. Sequence annotations were checked for frameshifts and manually refined with genome context information, multiple protein alignments (ClustalW), and identification of conserved protein domains using ScanProsite (http://us.expasy.org/prosite) and Pfam (www.sanger.ac.uk/Software/Pfam).

RESULTS

Growth on various P sources

Synechococcus WH 8103, like isolates from other genotypic clusters, is capable of using a variety of organic P sources (Fig. 1, Table 1), consistent with previous results for *Synechococcus* WH 7803 (Donald et al. 1997). The only exception is MIT S9220, a *Synechococcus* strain incapable of utilizing nitrate for growth (Moore et al. 2002), which is limited in the types of organic P that it can use for growth. *Prochlorococcus* HLI MED4 can grow on a wide variety of organic P sources (Table 1), consistent with findings for

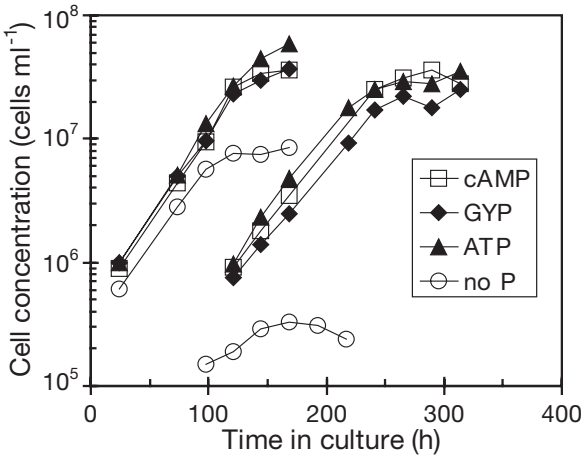

Fig. 1. Representative growth curves for *Synechococcus* isolate WH 8103 grown on cAMP (□), GYP (◆), and ATP (▲) for 2 transfers compared to response of cells when no P source was added to the media (○). Growth in the first transfer of the no P culture reflects the carryover of P_i from the standard growth conditions of the initial parent culture. Data similar to that presented here were obtained for all the isolates tested (for abbreviations of media, see 'Materials and methods')

Table 1. Growth capability of *Prochlorococcus* and *Synechococcus* strains (clades in parentheses) on a variety of inorganic and organic P sources. +: growth; –: no growth; +/–: inconclusive; nt: not tested

	MED4 (HLI)	MIT 9312 (HLII)	MIT 9313 (LLIV)	WH 8103 (III)	WH 8102 (III)	WH 8012 (I)	WH 7803 (V)	MIT S9220 (VII)
NaH_2PO_4	+	+	+	+	+	+	+	+
Tripolyphosphate (PPP_i)	+	+	+	nt	+	nt	nt	nt
β-glycerophosphate (GYP)	+	–	–	+	+	+	+	–
Glucose-6-phosphate (G6P)	+	–	–	nt	+	nt	+	–
ATP	+	+	+	+	+	+	+	–
cAMP	+	–	–	+	+/–	–	–	–
2-aminoethylphosphonate	–	–	–	nt	nt	nt	nt	nt

Prochlorococcus PCC 9511, which has an identical 16S rRNA gene sequence (Rippka et al. 2000). *Prochlorococcus* MIT 9312 and MIT 9313 can utilize ATP, with only a slight reduction in growth rate (data not shown), but not any other organic P sources that were tested. Utilization of cAMP appears to be limited to MED4 and WH 8103, whereas growth results for WH 8102 on cAMP could not be repeated consistently and, thus, are inconclusive. Consistent with previous studies (Donald et al. 1997), WH 7803 cannot utilize cAMP for growth. The ability to utilize cAMP suggests the presence of an exoenzyme capable of decyclizing cAMP, such as phosphodiesterase, followed by further hydrolysis with APase or 5′-nucleotidase. Alternatively, the cells may have the ability to take up cAMP directly (Bruns et al. 2003); however, a candidate nucleotide transporter has not been described for these cyanobacteria.

Alkaline phosphatase activity

To further characterize the P physiology of *Prochlorococcus* and *Synechococcus*, APA was assessed under P_i-replete and P-starvation conditions through the course of the growth cycle for the 3 strains of *Prochlorococcus* (MED4, MIT 9312, MIT 9313) and 2 strains of *Synechococcus* (WH 8102 and WH 7803). P starvation in the cultures was reached when growth was saturated, as indicated by a constant cell concentration and a concomitant drop in PO_4 concentration in the media to the detection limit of the method (<0.25 µM PO_4), as demonstrated by the results for *Prochlorococcus* MED4 (Fig. 2). When PO_4 is present in high concentrations, *Prochlorococcus* isolates MED4 and MIT 9313 exhibit low (~0.2 amol PO_4 $cell^{-1}$ h^{-1}) constitutive APA per cell, whereas MIT 9312 shows essentially none (Fig. 3A–C). The 2 *Synechococcus* isolates also exhibit low constitutive APA when grown in P_i-replete ASW (~0.02 amol PO_4 $cell^{-1}$ h^{-1}, Fig. 3D,E).

When the batch cultures became starved for P_i, the APA responded differently for the different cultures. MED4 exhibited a dramatic, 43-fold increase in APA (Fig. 3A). On the other hand, MIT 9312 and MIT 9313 showed small changes in their APA (8-fold increase and 2.4-fold decrease, respectively; Fig. 3B,C). Like MED4, the 2 *Synechococcus* isolates showed increased APA, but to differing degrees (Fig. 3D,E). WH 7803 exhibited a modest 4-fold increase over constitutive APA levels, whereas WH 8102 exhibited an increase in APA that was biphasic. During the phase of decelerating growth, the APA jumped 6-fold relative to the low constitutive P_i-replete level. At the onset of the P_i-starved stationary phase, APA increased another 4-fold to reach a maximum of 0.239 ± 0.015 amol PO_4 $cell^{-1}$ h^{-1} (Fig. 3E). For both WH 7803 and WH 8102 the external P_i concentration fell below detectable limits (0.25 µM) at least 1 generation before the saturated growth phase was achieved (data not shown); however, the maximum APA was not achieved until after

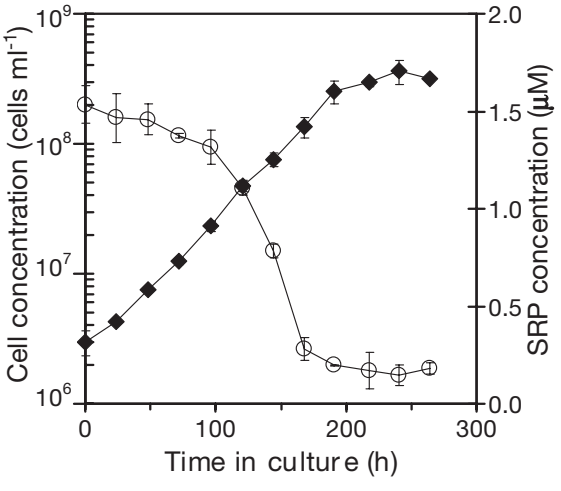

Fig. 2. Increase in cell concentration (◆) and decrease in SRP concentration (○) versus time for *Prochlorococcus* MED4 grown with PO_4 and used for APA measurements. Symbols and error bars represent average and standard deviation of replicate cultures. Data similar to that presented here were obtained for the other isolates used for APA measurements

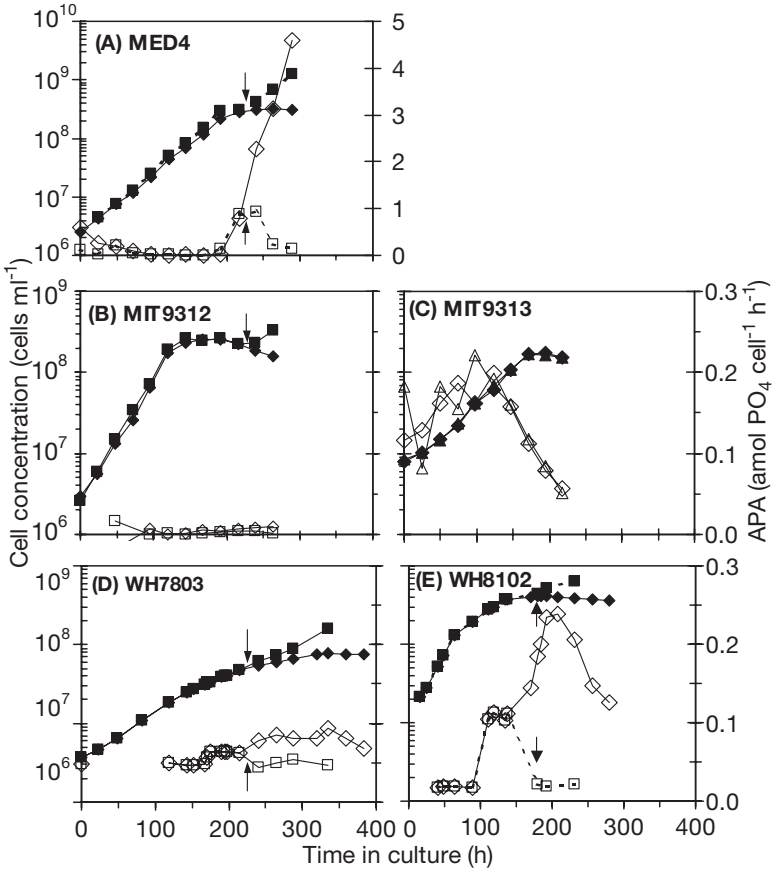

Fig. 3. Cell concentration (solid symbols) and cell-specific APA (open symbols) as a function of time for duplicate cultures of (A) *Prochlorococcus* MED4, (B) MIT 9312, (C) MIT 9313, and of (D) *Synechococcus* WH 7803 and (E) WH 8102. One replicate of each isolate (except MIT 9313) was rescued from P starvation by the addition of PO_4 (indicated by arrows); the square symbols represent the P-rescued culture. Note: the *y*-axes for MED4 are scaled differently from the others to account for higher cell yields and APA levels in this strain

saturation. The maximum measured APA per cell for both *Synechococcus* strains is similar to that measured in P-deplete cultures of *Synechococcus* sp. PCC7942 (Ray et al. 1991), and the rates of phosphate di-esterase activity (tested with the substrate bis-pNPP) were similar to rates of monoesterase activity at all time points.

The level of APA varies with the source and concentration of P. When MED4 is grown with organic P (GYP or ATP) as the sole source of P, the APA is 3 to 8 times higher than when PO_4 is available, suggesting slight up-regulation of APase expression or activity in the presence of external organic P (Fig. 4). APA further increased in these cultures upon reaching P starvation, however, we were not able to follow the ATP-grown MED4 cultures very far into the P-starvation stage. Although these same experiments were not carried out with the *Synechococcus* strains, when these strains are grown in SN media (Waterbury et al. 1986) containing 75% Sargasso Sea water base and 17.4 µM PO_4, APA was 2- to 3-fold higher (data not shown) than when grown with ASW and 10 µM PO_4, suggesting an increase in APA due to the likely presence of external organic P in the Sargasso Sea water. On the other hand, when PO_4 is added to P-starved cultures of MED4 (10 µM PO_4), WH 7803 and WH 8102 (50 µM PO_4 for rescuing the *Synechococcus* cultures), APA is repressed almost immediately (Fig. 3A,D,E), suggesting that high concentrations of PO_4 inhibit APA, the enzyme(s) is actively degraded, or gene expression is repressed.

Genomic comparisons

In order to comprehend the molecular basis of the specific differences in P physiology observed, we examined the completed genomic sequences of 6 marine cyanobacteria for genes related to P acquisition and regulation. We looked specifically for the presence and absence of genes encoding phosphate-uptake systems, outer-membrane porins, regulatory systems, and phosphate-scavenging pathways, such as extracellular phosphatases in 4 *Prochlorococcus* strains (MED4, MIT 9313, SS120, and MIT 9312) and 2 *Synechococcus* strains (WH 8102 and WH 7803) (Table 2). Unlike the much-studied phosphate-acquisition and -regulation system in *Escherichia coli*, these marine cyanobacterial genomes lack the genes associated with the low-affinity system

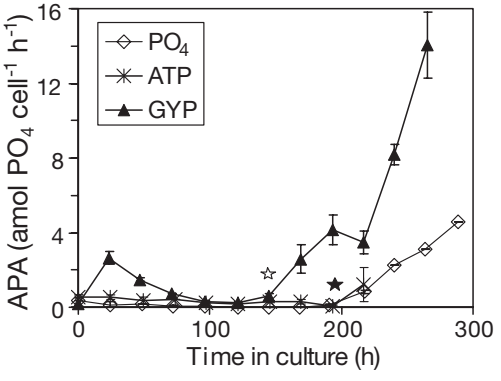

Fig. 4. MED4 average cell-specific APA as a function of time over the course of growth on PO_4 (◇), ATP (cross-hatched symbols), and GYP (▲). The stars indicate the point at which the cultures entered the stationary growth phase. The open star is for GYP cultures; the solid star is for both the PO_4 and ATP cultures. Symbols and error bars represent average and standard deviation of replicate cultures

Table 2. Phosphorus assimilation and regulatory genes present in unicellular marine cyanobacterial genomes (+: presence of the gene; –: gene is absent). The genome copy number for all genes present in the genomes is 1, except those with multiple copies indicated by the number in parentheses

Gene function	Gene name	Reference sequence accession no.[a]	Syn WH 8102	Syn WH 7803	Pro MED4	Pro MIT 9312	Pro MIT 9313	Pro SS120
Low-affinity P transporters	*pitA,B*	NP_417950 NP_417461	–	–	–	–	–	–
Outer membrane porin	*phoE*	AAG54566	–	–	–	–	–	–
High-affinity P_i-binding protein	*pstS*	CAA50495	+ (4)	+ (3)	+	+	+ (2)	+ (2)
P_i-channel components	*pstA,B,C*	NP_897363 NP_897364 NP_897365	+	+	+	+	+	+
P-limitation-inducible porins	*som*	NP_898316	+ (4)	+ (5)	+ (3)	+(5)	+ (4)	+ (2)
Phosphonate ABC-type transport system for phosphonates	*phnC,D,E*	NP_897263 NP_897262 NP_897261	+	+	+	+	+	+
Phosphonate operon regulator	*phnO*	AAG59293	–	–	–	–	–	–
Phosphonate biodegradation	*phnF-N,P*	*Nostoc* sp. PCC 7120[b]	–	–	–	–	–	–
Alkaline phosphatase (APase)	*phoA*	NP_898480 NP_892896	+ (2)	+	+[c]	+[c]	–	–
5'-nucleotidase	*5ND* (*ushA*)	NP_898471	+	–	–	–	–	–
APase-like	*dedA*	NP_896215	+	+	+	+	+	+
Exopolyphosphatase	*ppX* (*gppA*)	NP_897937	+	+	+	+	+	+
Polyphosphate utilization (kinase)	*ppK*	NP_898584	+	+	+	+	+	+
P_i sensor kinase	*phoR*	NP_897041	+	+	+	+	–[d]	–
Response regulator	*phoB*	NP_897040	+	+	+	+	+	–
Modulator of P_i transduction	*phoU*	NP_440013	–	–	–	–	–	–
Potential transcriptional regulator	*ptrA*	CAA87409	+	+	+	–	–	+
P_i-starvation-inducible protein (possible RNA helicase)	*phoH*-like	NP_897709	+	+	+	+	+	+
P_i-starvation-inducible protein	*psip1*	NP_896260	+	–	+	–	–	–

[a]The significance of BLASTP matches against the reference sequence in the third column are supported by E-values <1e-55. The absence of a gene sequence is supported by E-values >0.05

[b]No similar sequences were found when phosphonate metabolism genes from *Nostoc* sp. PCC 7120 and *E. coli* were used as reference

[c]MED4 and MIT 9312 PhoA have amino acid identity of 91%

[d]The gene sequence is present but contains frameshifts and/or deletions

(*pitAB*) and homologues of the P-specific porin (*phoE*). However, the marine cyanobacteria contain orthologues of many components of bacterial high-affinity P-uptake systems, most notably the high-affinity periplasmic binding protein and ABC-type transport system (PstSCAB), and a number of P-limitation-inducible outer-membrane porins (Som proteins). An interesting feature of marine cyanobacterial genomes is the multiplication of the *pstS* and *som* genes, which are present in multiple, nonidentical copies in all strains, with the exception of a single copy of *pstS* in MED4 and MIT 9312 (Table 2). In addition to the Pst ABC–type transport system, the genes for a putative phosphonate ABC transporter system, *phnCDE* (Palenik et al. 2003), are also conserved within the genomes studied, indicating the potential to at least

actively transport phosphonates into the cell. However, on the basis of similarity searches, we were unable to identify genes encoding enzymes (PhnF-N,P) capable of degrading phosphonate (carbon–phosphorus) bonds or the phosphonate-operon-regulatory protein, PhnO. The lack of phosphonate-degrading enzymes is consistent with the inability of *Prochlorococcus* isolates to grow with AEP. Testing additional phosphonate compounds is necessary to more fully understand the phosphonate-related physiology of *Prochlorococcus* and to confirm the previous report that WH 8102 is capable of growing on an unidentified phosphonate source (Palenik et al. 2003).

Apart from the potential ABC-transport system for phosphonates, no genes were identified for the transport of organic phosphates in these marine cyanobacterial genomes. This is also the case for numerous freshwater cyanobacteria that are reliant on the synthesis of periplasmic phosphatases to enable P_i scavenging through hydrolysis of organic phosphates under P_i-deficient conditions (Grossman et al. 1994). Gene sequences similar to characterized APases (such as *phoA*) were identified in HL *Prochlorococcus* MED4 and MIT 9312, in *Synechococcus* WH 8102 and WH 7803, but not in LL *Prochlorococcus* ecotypes MIT 9313 or SS120 (Table 2). The *phoA* gene sequence for MIT 9312 has the highest sequence identity to that of the other HL *Prochlorococcus* MED4, which has a deduced amino acid (aa) sequence (760 aa, 83 kDa predicted molecular weight) most similar to the predicted 79 kDa APase of *Silicibacter* sp. TM1040 (Fig. 5). This suggests that the HL *Prochlorococcus* may have acquired their *phoA* gene in a lateral gene transfer event. In contrast, *Synechococcus* WH 8102 and WH 7803 possess different complements of *phoA*. The WH 7803 genome encodes 1 *phoA* gene sequence (Fig. 5) with a predicted mass of 185 kDa homologous to an atypical 145 kDa APase, which is responsible for increased APA in *Synechococcus* sp. PCC 7942 (Ray et al. 1991) and similar to the 149 kDa P-limitation-induced APase found in *Synechocystis* PCC6803 (Hirani et al. 2001). Strain PCC 7942 also contains a second constitutive (noninducible) APase of 61 kDa, designated as *phoV* (Wagner et al. 1995) for which no obvious homologues have been found within the genomes examined in this study. In contrast to WH 7803, WH 8102 encodes at least 3 APases (Palenik et al. 2003): an APase (749 aa, 80 kDa) similar to sequences found in γ-proteobacteria (*Shewanella* and *Vibrio*) and 2 adjacent APase genes, a predicted APase (576 aa, 63 kDa) and a predicted APase/5′nucleotidase (750 aa, 80 kDa), both of which are homologous to the C-terminal of the WH 7803 *phoA* (Fig. 5). A small (~200 aa), putative APase-like gene, *dedA*, named for its predicted transmembrane region, is also found in all the marine cyanobacterial genomes examined for this study; however, its specific function is not known.

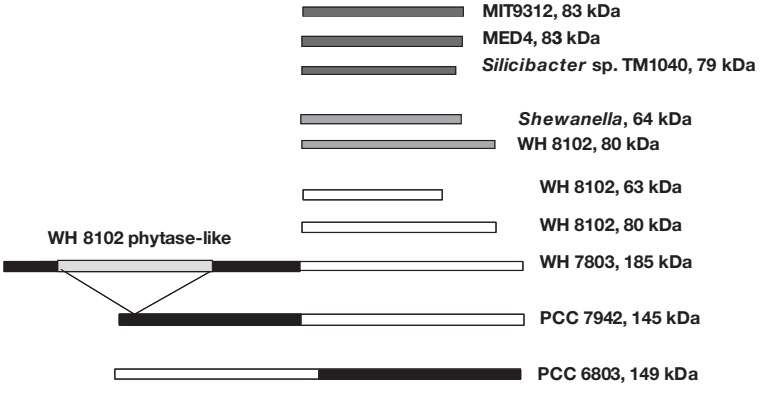

Fig. 5. Simplified alignment of domains in *phoA* and *phoA*-like genes found in marine cyanobacterial and proteobacterial genomes with high sequence similarities, indicated by matched shading. Lengths of rectangles represent relative protein sizes. Predicted molecular weights (in kDa) for each protein are indicated

There are also obvious differences in gene complement with respect to phosphate sensing and regulation amongst the genomes (Table 2). The HL *Prochlorococcus* (MED4 and MIT 9312) and the 2 marine A *Synechococcus* (WH 8102 and WH 7803) contain homologues of the *phoR* gene, encoding a sensor histidine kinase, and the *phoB* gene, encoding the cognate response regulator responsible for P sensing and regulation in a wide variety of bacteria (e.g. see Wanner 1996). LL-adapted *Prochlorococcus* MIT 9313 contains a *phoB* gene, but a nonfunctional *phoR* (Scanlan & West 2002), whereas *Prochlorococcus* SS120 appears to lack both regulatory genes. The gene corresponding to PtrA, a putative transcriptional activator found in WH 7803 (Scanlan et al. 1997a), is also found in WH 8102, MED4, and SS120, but is missing from MIT 9312 and MIT 9313. Another regulatory gene encoding for a modulator of P_i transduction, *phoU*, is also missing from all the marine cyanobacterial genomes, but is found in the freshwater cyanobacterium *Synechocystis* PCC6803 (www.kazusa.or.jp/cyanobase/).

Other genes involved in P acquisition are also present in all 6 marine cyanobacterial genomes, including the genes involved in internal polyphosphate metabolism (*ppK* and *ppX*), consistent with the ability of the strains tested to grow with PPP_i as the sole source of P (Table 1), and the P-starvation-induced protein, *phoH*, believed to be an RNA helicase that is highly conserved in bacteria (Kim et al. 1993). Another gene for a P-starvation-induced protein, *psip1* (N. J. West & D. J. Scanlan unpubl. data), is found in MED4 and WH 8102, but not in MIT 9312, SS120, MIT 9313, and WH 7803.

DISCUSSION

Utilization of organic P sources

The ability to utilize organic sources of phosphate varies widely among the strains of *Prochlorococcus* and *Synechococcus* tested so far. Some of the variability can be explained by presence and/or absence of P-acquisition and -scavenging genes. *Synechococcus* isolates from several genotypic clusters are capable of using a variety of organic P sources (Table 1), as was demonstrated previously for *Synechococcus* WH 7803 (Donald et al. 1997). Consistent with this ability is the presence of essential genes required for acquisition and regulation of P_i under phosphate-deficient conditions. One exception is MIT S9220, a *Synechococcus* strain incapable of utilizing nitrate for growth (Moore et al. 2002), which cannot utilize any of the organic P compounds tested. However, the genome is not available for this strain of marine *Synechococcus*. Like *Synechococcus* strains WH 8102 and WH 7803, HLI *Prochlorococcus* MED4 is capable of utilizing a broad range of organic P compounds and contains a similar suite of P-acquisition and -regulation genes. Thus, these genotypes may have an advantage in P_i-depleted marine waters that have greater concentrations of organic P compounds due to increased N_2 fixation and P_i depletion (Bjorkman et al. 2000).

Like freshwater cyanobacteria, marine cyanobacteria appear to be reliant on the expression of exported phosphatases, such as APase, for hydrolysis of phosphate from organic sources during periods of P_i restriction. Indeed, WH 8102, WH 7803, and MED4 have at least 1 gene for APase, *phoA*, as well as the putative APase-like gene, *dedA*, consistent with their ability to utilize various organic phosphates. Interestingly, HLII *Prochlorococcus* MIT 9312 contains a very similar P-acquisition gene compliment to HLI MED4, with no obvious mutations, but only utilizes ATP as an organic P source. The only P-related genes that MIT 9312 appears to lack relative to MED4 are *psip1* and *ptrA*. On the other hand, the LL *Prochlorococcus* strains MIT 9313 and SS120 lack *phoA*, but contain *dedA*, which may explain the ability of MIT 9313 to utilize ATP, or ATP may be taken up into the periplasm through one of the many P-limitation-induced porins.

Alkaline phosphatase activity

The ability to utilize a wide variety of organic P sources and the presence of APases in MED4, WH 8102, and WH 7803 are reflected in their APA. These 3 cyanobacteria express constitutive APA when PO_4 is present, and then increase their APA when PO_4 becomes depleted and the cells become P-starved. Unexpectedly, HLII MIT 9312, which contains essentially the same *phoA* as MED4, does not exhibit constitutive APA and only shows minimal increases in APA once it becomes P starved. Possible explanations include the lack of Psip1, which may be required for constitutive APA, and/or the lack of PtrA, which may be required for activation or enhancement of expression of genes responding to P limitation, such as *phoA* and *pstSCAB*. The LLIV *Prochlorococcus* ecotype MIT 9313, which cannot utilize many organic P compounds and does not contain a *phoA* equivalent, exhibits very low but measurable constitutive APA. The expression of measurable APA in this strain is possibly due to the presence of the APA-like DedA protein, though gene-expression studies are required to pin this down. The frameshift and mutation in *phoR* of MIT 9313 (Scanlan & West 2002) suggest that this LL strain lacks the ability to regulate a response to P depletion, possibly explaining the decrease in cell-specific APA once P_i becomes depleted. The total absence of *phoB* and *phoR* in SS120 implies that this LL strain also is incapable of regulating a response to P depletion, but determining this awaits an axenic strain of SS120, which currently is not available.

Synechococcus WH 7803 and WH 8102 exhibit similar low constitutive levels of APA when P replete (>50 µM PO_4). However, after successive subculture in ASW with 10 µM PO_4, WH 7803 displayed a 2-fold increase in baseline AP activity, while WH 8102 maintained activity at constitutive levels. This slight difference in the pattern of APA induction hints that these strains could sense P limitation at different threshold concentrations of P_i or could possess different mechanisms of APA regulation. The maximum APA activity for WH 7803 and WH 8102 was not achieved until at least 120 h after the external P concentration had fallen below detectable levels. The time delay in reaching maximum APA may be due simply to a rather high detection limit for P. However, another explanation for this observation is in line with the response of *Synechocystis* PCC6803, where 1 of 2 operons encoding the high-affinity P-uptake system PstSCAB is expressed during the early stages of P depletion, while the expression of *phoA*, *nucH* (extracellular nuclease), and the second *pst* operon occurs later (Suzuki et al. 2004), suggesting a distinction between P-limitation (expression of *pstSCAB*) and P-starvation responses (expression of *pstSCAB* and APA). A maximum APA was not observed for MED4 or MIT 9312, though these cultures were not followed beyond ~100 h after P starvation was reached.

Another intriguing observation is that MED4, WH 8102, and WH 7803 express higher APA activity when grown in the presence of organic P. This occurs in the

Synechococcus cultures even when the external P_i concentration is relatively low (<25 µM), but not preventing growth, suggesting that these strains are capable of regulating their APA in order to exploit organic and inorganic sources of P simultaneously, as observed for marine microbial communities (Bjorkman & Karl 2003). Furthermore, the lack of a constitutive low-affinity P-uptake system (Pit system) suggests that the high-affinity, energy-dependent ABC transporter is always required for P uptake in the marine environment. The possession of multiple copies of the periplasmic phosphate-binding protein, possibly under differential P regulation, in HL *Prochlorococcus* and *Synechococcus* WH 8102 and WH 7803 may serve to provide a rapid response to short periods of P limitation. Since the energy requirements for P_i uptake are supplied by photosynthesis (Ritchie et al. 2001), the ability to opportunistically use organic P at the same time would provide a significant advantage over heterotrophic competitors.

Ecological implications

Apart from the APase-like *dedA* genes present in all of the strains examined in this study, WH 8102, WH 7803, and the HL *Prochlorococcus* do not possess orthologous APase genes. In other words, the current array of APase genes among the genomes was not inherited from a common ancestor, implying that APase genes were acquired independently through horizontal gene transfer (as appears to be the case for *phoA* in the 2 HL *Prochlorococcus*) and/or loss of ancestral genes. Horizontal gene transfer is supported by the low mol%G+C composition of *phoA* sequences relative to the genome average mol%G+C in WH 7803 and WH 8102. Furthermore, the selection of different APase genes in each strain may reflect the total range of organic substrates encountered in their respective ecological niches. In general APases and 5′-nucleotidases are capable of hydrolyzing a wide range of organic P monoesters (Wagner et al. 1995, Wanner 1996). Although the substrate specificity of the APases from PCC7942 or PCC6803 have not been examined in great detail, these proteins retain domains conserved amongst alkaline phosphatases such as multiple P-loop motifs, which are thought to be involved in binding terminal PO_4 moieties and could serve to extend the range of organic P substrates hydrolyzed (Ray et al. 1991, Hirani et al. 2001). In this regard the WH 7803 *phoA* is unusual in that it contains a 480-aa insertion in the conserved N-terminal ATPase domain of its 185-kDa PhoA (Fig. 5). The inserted sequence is homologous with the first 580 aa of a conserved hypothetical protein found in WH 8102, which itself displays some similarity to phytase (*myo*-inositol hexakisphosphatase), an enzyme that hydrolyzes phytic acid or similar complex organic P-rich substrates. The presence of this unusual insertion sequence and gene raises the intriguing possibility that complex organic P-rich substrates of terrestrial origin may provide an advantage to some *Synechococcus* in coastal marine environments, even though phytic acid has not been identified as a component of dissolved organic phosphorus in recent coastal and open ocean surveys (Monaghan & Ruttenberg 1999, Kolowith et al. 2001).

The P physiology presented in this study is consistent in part with the ecological niche distribution of marine cyanobacteria. LL *Prochlorococcus* MIT 9313 has a limited capacity to utilize organic P sources and expresses extremely low levels of APA, and 2 LL *Prochlorococcus* (MIT 9313 and SS120) lack an obvious *phoA* gene and contain mutations or deletions in key P-regulatory genes, such that a P-stress response is likely not regulated. These deficiencies in P acquisition and regulation may reflect the fact that relatively high and constant inorganic phosphorus levels are found in the deep euphotic zone, where LL *Prochlorococcus* reside (Scanlan & West 2002, Fuller et al. 2005), and precise regulation of the P-acquisition machinery would be unnecessary. In contrast, HL *Prochlorococcus* and *Synechococcus* occupy the upper photic zone, where inorganic nutrients, such as P_i, are typically depleted and competition for recycled organic P is great (Bjorkman & Karl 1994, Li et al. 1998, Fuller et al. 2005).

However, the results from this study and recent field observations are not entirely consistent with this simplistic model. HLII *Prochlorococcus* MIT 9312, which does not utilize a variety of organic P sources and does not exhibit significant levels of APA even under conditions of P starvation, would appear to be at an ecological disadvantage in P-limited surface waters compared to HLI *Prochlorococcus* MED4. Yet, the HLII genotype dominates natural *Prochlorococcus* populations in nutrient-depleted summer surface waters of the Red Sea (Fuller et al. 2005), the North Pacific (E. Zinser & S. W. Chisholm pers. comm.), and the Sargasso Sea (Scanlan & West 2002), implying that it may be unaffected by P limitation in contrast to other HL *Prochlorococcus* genotypes, possibly due to more efficient P_i uptake than the HLI type, which could be due to, and reflected in, differences in gene regulation. This hypothesis awaits confirmation.

The results reported here have important implications for the use of bulk APA as an indicator of the P status of phytoplankton communities. We have shown that APA is expressed constitutively in several strains of *Synechococcus* and *Prochlorococcus* and the presence of organic P sources and conditions of P limitation

and starvation elicit markedly different capacities to express APA and utilize organic P sources. Genomic comparisons make it clear that APA may be attributed to multiple enzymes with different regulation and substrate specificities in the *Prochlorococcus* and *Synechococcus* strains studied. It is important to note that the use of different markers for APA in this study (pNPP and MUFP) may add other confounding factors, such as differential availability of each enzyme for each substrate. Consequently, bulk APA measurements may not reflect community-wide P status, but rather P limitation of undetermined subpopulations of the phytoplankton community. Clearly more work is needed in order to define genotype-specific responses to general P stress in marine picophytoplankton, a task that will benefit greatly from in-depth analysis of diverse axenic isolates and on-going genome-sequencing projects, as well as further use and optimization of methodologies that are capable of assessing P status at the single-cell level (Scanlan et al. 1997b, Dyhrman et al. 2002). Understanding the nutrient-stress response of specific eco/genotypes of marine picophytoplankton is critical to being able to determine the *in situ* nutrient status of these organisms, particularly in response to climate-related environmental changes. And more generally this work suggests that studies of nutrient enrichment on natural populations (e.g. ocean fertilization experiments) need careful interpretation given that other phytoplankton groups also likely share a plethora of eco/genotypic responses to nutrient limitation.

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