ORIGINAL ARTICLE

Genomic potential for arsenic efflux and methylation varies among global *Prochlorococcus* populations

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The globally significant picocyanobacterium *Prochlorococcus* is the main primary producer in oligotrophic subtropical gyres. When phosphate concentrations are very low in the marine environment, the mol:mol availability of phosphate relative to the chemically similar arsenate molecule is reduced, potentially resulting in increased cellular arsenic exposure. To mediate accidental arsenate uptake, some *Prochlorococcus* isolates contain genes encoding a full or partial efflux detoxification pathway, consisting of an arsenate reductase (*arsC*), an arsenite-specific efflux pump (*acr3*) and an arsenic-related repressive regulator (*arsR*). This efflux pathway was the only previously known arsenic detox pathway in *Prochlorococcus*. We have identified an additional putative arsenic mediation strategy in *Prochlorococcus* driven by the enzyme arsenite S-adenosylmethionine methyltransferase (ArsM) which can convert inorganic arsenic into more innocuous organic forms and appears to be a more widespread mode of detoxification. We used a phylogenetically informed approach to identify *Prochlorococcus* linked arsenic genes from both pathways in the Global Ocean Sampling survey. The putative arsenic methylation pathway is nearly ubiquitously present in global *Prochlorococcus* populations. In contrast, the complete efflux pathway is only maintained in populations which experience extremely low $\text{PO}_4\text{:AsO}_4$, such as regions in the tropical and subtropical Atlantic. Thus, environmental exposure to arsenic appears to select for maintenance of the efflux detoxification pathway in *Prochlorococcus*. The differential distribution of these two pathways has implications for global arsenic cycling, as their associated end products, arsenite or organoarsenicals, have differing biochemical activities and residence times.

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Introduction

The marine picocyanobacterium *Prochlorococcus* is the dominant phytoplankton in oligotrophic tropical and subtropical oceans (Campbell *et al.*, 1997; DuRand *et al.*, 2001; Scanlan, 2012). In the subtropical gyres, surface phosphate concentrations are extremely low and in some cases may limit primary production (Wu *et al.*, 2000). *Prochlorococcus* has evolved several adaptations to this low P environment, including a high specific affinity for phosphate uptake and low cellular P quotas (Krumhardt *et al.*, 2013). These low cellular P quotas are achieved in part by their small genome sizes (Rocap *et al.*, 2003) and predominance of sulfolipids over phospholipids in the membrane (Van Mooy *et al.*, 2006). As a result, *Prochlorococcus* under P-replete conditions have a cellular N:P content over 20, well above the Redfield ratio of 16:1, and can exceed 100:1 under P starvation (Bertilsson *et al.*, 2003; Haldal *et al.*, 2003).

Prochlorococcus can be divided up into physiologically and genetically distinct ecotypes (Moore

et al., 1998) which reflect adaptations to environmental parameters like light intensity (Moore *et al.*, 1998; Moore and Chisholm, 1999), temperature optima (Johnson *et al.*, 2006) and nitrogen utilization capabilities (Moore *et al.*, 2002) among others. The multiple strains of *Prochlorococcus* possess a variable assortment of accessory genes suited to the specific environments they inhabit (Rocap *et al.*, 2003; Kettler *et al.*, 2007). The complement of phosphorus acquisition genes in *Prochlorococcus* also varies among the strains (Martiny *et al.*, 2006). Many of these genes are found on genomic islands (Coleman *et al.*, 2006) and some have been acquired by horizontal transfer (Rocap *et al.*, 2003). Thus, the occurrence of phosphorus acquisition genes is not congruent with 16S rRNA phylogeny (Martiny *et al.*, 2006), but can be correlated with phosphate concentrations in the regions these strains were isolated from. In the field, *Prochlorococcus* populations in phosphorus-scarce regions contain a greater number of phosphorus acquisition genes, indicating the selective force of nutrient availability on *Prochlorococcus* genetic capacity (Martiny *et al.*, 2009).

The physiochemical similarities between inorganic phosphate and arsenate can result in the indiscriminate uptake of toxic arsenic into cells by cellular phosphate acquisition systems. The high affinity phosphate uptake system ubiquitously

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present among *Prochlorococcus* strains (Figure 1) consists of the periplasmic binding protein PstS and the membrane-bound ABC-type transporter PstCAB (Scanlan *et al.*, 2009). The PstCABS system is unable to completely differentiate between arsenate and phosphate in *Escherichia coli* (Rosenberg *et al.*, 1977; Tawfik and Viola, 2011). Once inside the cell, arsenate can become toxic by competing with phosphate, for example, through the decoupling of oxidative phosphorylation, the process that produces ATP (Mandal and Suzuki, 2002; Oremland and Stolz, 2003). The reduced form, arsenite, is even more toxic because it interferes with enzyme activity by bonding to $-SH$ and $-OH$ groups in enzymes (Mandal and Suzuki, 2002; Akter *et al.*, 2005).

Microbes have evolved multiple mechanisms for cellular defense against arsenic, and the genes involved are taxonomically widespread and subject to frequent horizontal transfer (Stolz *et al.*, 2006; Páez-Espino *et al.*, 2009). The general efflux detoxification pathway involves the reduction of arsenate to arsenite, and then subsequent expulsion of arsenic from the cell through arsenite-specific transporters (Carlin *et al.*, 1995; Ghosh *et al.*, 1999). The efflux system, which has evolved independently at least two different times, consists of an arsenate reductase (the analogs ArsC, ACR2 and wzb-like low molecular weight protein tyrosine phosphatase (Bennett *et al.*,

2001)) and an arsenite-specific efflux pump (ArsB or ACR3) (Páez-Espino *et al.*, 2009). Additional arsenic-related genes include an ATPase (ArsA) that couples with ArsB for the expulsion of arsenite from cells, the regulatory elements ArsR and ArsD, and a gene of unknown function *arsH* (Stolz *et al.*, 2006). The genes sufficient for a complete efflux pathway were previously identified in *Prochlorococcus* genomes (Scanlan *et al.*, 2009; Figure 1) including the arsenate reductase, *arsC*, the *acr3* arsenite efflux transporter and *arsR*, an arsenite-binding *trans*-acting repressive regulator (Xu *et al.*, 1996). This efflux detoxification was believed to be the major arsenic detoxification strategy for *Prochlorococcus*.

A second microbial arsenic detoxification strategy is the methylation of arsenic into more innocuous organic compounds. This pathway involves the repeated methylation and reduction of arsenical compounds to less toxic states mediated by the enzyme ArsM, arsenite S-adenosylmethyltransferase (Figure 1) (Qin *et al.*, 2006; Yin *et al.*, 2011). In *Rhodospseudomonas palustris*, the methylation pathway includes the intermediates monomethylarsonate, and dimethylarsonate, and ultimately results in the production of the gas trimethylarsine oxide, TMAO(g), and subsequent release of arsenic from the cell (Qin *et al.*, 2006). However, the end products of the methylation pathway vary across taxa, and it is not currently possible to determine which organoarsenicals are synthesized through genetic analysis alone (Dembitsky and Levitsky, 2004). TMAO(g) production and presence of *arsM* genes have been observed in three freshwater cyanobacteria, *Microcystis* sp. PCC7806, *Nostoc* sp. PCC7120 and *Synechocystis* sp. PCC6803 (Yin *et al.*, 2011). A putative *arsM* was identified in the picocyanobacterium *Synechococcus* sp. WH 8102 based on sequence similarity and protein binding domain homology (Thomas *et al.*, 2010), but it has not been demonstrated whether or not this species is capable of methylating arsenic in the lab.

In the marine environment, inorganic arsenate accounts for about 85% of the standing pool of arsenic with the remaining portion of the arsenic pool comprised of the inorganic arsenite and various organoarsenicals, including two of the main products of the methylation pathway, monomethylarsonic acid and dimethylarsinic acid (Andreae, 1979; Cutter *et al.*, 2001; Cutter and Cutter, 2006; Wurl *et al.*, 2013). Arsenate has a nutrient-like depth distribution because it is a bioactive molecule which is transformed by marine biota in the surface waters *via* the two pathways described above (Andreae, 1979). The degree of uptake and subsequent transformation of arsenate is likely affected by the standing phosphate concentration (Cutter *et al.*, 2001; Cutter and Cutter, 2006). In oligotrophic gyres, the ratio of soluble reactive phosphorus to arsenate can shift dramatically as phosphorus is depleted, resulting in a greater abundance of arsenate relative

Figure 1 Diagram representing an idealized cross-section of a *Prochlorococcus* cell highlighting mechanisms likely responsible for arsenic entry into the cell and detoxification. (1) Arsenate likely enters the cell through the inorganic high affinity phosphate transporters PstCABS. (2) Once arsenate is inside the cytoplasm it is reduced by the arsenate reductase ArsC to the (III) oxidation state, arsenite. (3) Arsenite can then bind to the *trans*-acting ArsR-repressive regulator, (4) arsenite can be recognized by the arsenite efflux transporter ACR3 which shuttles the toxin out of the cell, or (5) the arsenite can be methylated by the putative arsenite S-adenosylmethyltransferase, ArsM. Monomethylarsonic acid (MMA) can undergo a series of oxidation and methylation steps to further methylate the species to dimethylarsinic acid (DMA); further methylation into more compound organoarsenicals may also be possible, but cannot be confirmed without laboratory analysis.

to available phosphate (Karl and Tien, 1997; Wurl *et al.*, 2013). While inorganic arsenic concentrations are relatively stable throughout the surface waters of the open ocean ranging from 12.9–15.7 nmol l⁻¹ (Andreae, 1979; Cutter and Cutter, 1998; Cutter *et al.*, 2001; Ellwood and Maher, 2002; Cutter and Cutter, 2006), surface phosphate concentrations are far more variable, ranging from <0.1 µmol l⁻¹ in oligotrophic subtropical gyres to >2.0 µmol l⁻¹ in the Southern Ocean (Karl, 2007). Thus, the ratio of available PO₄:AsO₄ also varies widely across different ocean regions, and may be a major factor driving the risk of competitive arsenic uptake.

To better understand arsenic transformations in marine cyanobacteria, we analyzed arsenic-related genes in *Prochlorococcus* genomes, and identified a putative arsenite S-adenosylmethyltransferase. The evolutionary history of the marine picocyanobacterial arsenic genes was inferred through phylogenetic analysis. To further explore the biogeochemical connection between phosphate scarcity and arsenic transformations in marine ecosystems, we used a phylogenetically informed approach to identify *Prochlorococcus*-linked arsenic-related genes from the Global Ocean Sampling (GOS) metagenomic dataset spanning various ocean nutrient regimes (Venter *et al.*, 2004; Rusch *et al.*, 2007). The goal of this analysis was to elucidate the putative arsenic-related mechanisms used by *Prochlorococcus*, and to understand the environmental selective pressures driving the existence of these mechanisms.

Materials and methods

Identification of *Prochlorococcus* arsenic genes

Arsenic efflux pathway detoxification genes (*arsR*, *arsD*, *arsA*, *arsB*, *acr3*, *arsC* and *arsH*) were queried with blastp searches of the *Prochlorococcus* (taxaid: 1218)

portion of NCBI's non-redundant protein sequence database (Altschul *et al.*, 1990) using arsenic detoxification genes from other organisms as queries (Páez-Espino *et al.*, 2009). For genes identified in *Prochlorococcus* genomes (*arsR*, *arsC*, *acr3*) as well as the single copy core genes *glnA*, *rpsD* and *tyrS*, the Clusters of Orthologous Groups (COG) associated with blast results were identified (Tatusov *et al.*, 2000; Table 1). The genome browser tool on MicrobesOnline.org (Dehal *et al.*, 2009) was used for identifying genomic context of the various genes within the picocyanobacteria.

To identify a *Prochlorococcus* *arsM*, the COGs associated with known functional *arsM* sequences from other organisms (Qin *et al.*, 2006; Yin *et al.*, 2011) were obtained (COGs 500 and 2226). Sequences of the closely related SAM-dependent mycolic acid cyclopropane synthetase (*cmsA*) were also included as an outgroup (Yuan and Barry, 1996).

Secondary structure prediction was used to confirm homology of the putatively identified *arsM* sequence in *Prochlorococcus*. Sequences identified as *arsM* in the freshwater cyanobacteria *Microcystis* sp. PCC7806, *Nostoc* sp. PCC7120, and *Synechocystis* sp. PCC 6803 (GenBank accession numbers HQ891147, HQ891148, and HM776638, respectively) were aligned with the putatively identified *arsM* sequence in *Prochlorococcus* MED4. All four sequences were submitted individually to the Phyre2 protein prediction server (Kelley and Sternberg, 2009).

Reference alignments and phylogenetic trees

Sequence collection was performed by searching a list of reference organisms (including cyanobacteria, abundant marine bacteria in the GOS metagenomic set (Rusch *et al.*, 2007) and bacterial strains with previously identified arsenic detoxification genes (Páez-Espino *et al.*, 2009)) for genes associated with

Table 1 Presence/absence of arsenic detoxification genes identified within genomes of *Prochlorococcus* strains

Strain	Clade	Isolate Region	<i>arsR</i>	<i>acr3</i> ^a	<i>arsC</i>	<i>arsM</i>
			Regulator	Efflux transporter	Reductase	Methyltransferase
			COG640	COG798	COG1393	COG500
MED4	High Light 1	Med. Sea	PMM0714	PMM0716	PMM0512	PMM0416
MIT9515	High Light 1	Eq. Pacific	—	—	P9515_05761	P9515_04771
AS9601	High Light 2	Arabian Sea	—	—	A9601_05691	A9601_04661
MIT9301	High Light 2	Sargasso Sea	P9301_12351	P9301_12581	P9301_05391	P9301_04351
MIT9215	High Light 2	Eq. Pacific	—	—	MIT9215_706	MIT9215_808
MIT9312	High Light 2	Gulf Stream	PMT9312_0726	—	PMT9312_0513	PMT9312_0411
NATL2A	Low Light 1	N. Atlantic	PMN2A_0446	PMN2A_0447	PMN2A_1845	—
NATL1A	Low Light 1	N. Atlantic	NATL1_11581	NATL1_11591	NATL1_05701	—
SS120	Low Light 2	Sargasso Sea	Pro1578	—	Pro0511	—
MIT9211	Low Light 3	Eq. Pacific	—	—	P9211_05131	P9211_04141
MIT9313	Low Light 4	Gulf Stream	PMT1001	—	PMT1256	PMT1171
MIT9303	Low Light 4	Sargasso Sea	P9303_11041	P9303_11311	P9303_07481	P9303_08531

Abbreviation: COG, Clusters of Orthologous Groups. Presence of gene marked by locus tag, absence represented by '—'. Associated COGs for relevant genes also denoted. *Prochlorococcus* genomes are listed in order of physiological light optima which follows the 16S rRNA phylogeny.

^a*acr3* referred to as '*arsB*' in Scanlan *et al.*, (2009) and as '*arsA*' in Martiny *et al.* (2006).

relevant COGs (Table 1) using the online portal www.MicrobesOnline.org (Dehal *et al.*, 2009). The ortholog gene tree tool on www.MicrobesOnline.org was used to generate a list of additional sequences of broader taxonomic range to add greater phylogenetic resolution.

Nucleic acid sequences of the genes were downloaded from www.MicrobesOnline.org and aligned using TranslatorX v 1.1 (Abascal *et al.*, 2010). The amino acid-translated alignments were then used to build phylogenetic reference trees using the program RAXML v 7.2.8 (Stamatakis, 2006). A maximum likelihood tree was inferred taking the best of 20 starting trees using the amino acid substitution matrix model which resulted in the best likelihood score during a trial run (*arsR*: WAGF; *acr3*: WAGF; *arsC*: WAGF; *arsM*: VTF; *glnA*: RTREVF; *rpsD*: RTREVF; *tyrS*: WAG), empirical character frequencies and a gamma model of rate heterogeneity with RAXML estimated alpha value. Bootstrap analyses were conducted on all trees at $n=100$. These trees, which contained hundreds of taxa (*arsR*: 295; *acr3*: 112; *arsC*: 105; *arsM*: 289; *glnA*: 207; *rpsD*: 99; *tyrS*: 110), were used for phylogenetic placement of metagenomic reads.

For ease of viewing the broader phylogenetic relationships within the gene trees, smaller trees were constructed. For the genes *arsR*, *arsC* and *acr3*, a subset of sequences representing the major phylogenetic groups from the original tree were used to construct a smaller tree. For *arsM*, the phylogenetic tree was focused on the clades containing taxa with known arsenite-methyltransferase activity. In addition, arsenite-methyltransferases from eukaryotic algae and mammals were obtained from the OrthoMCL database, which groups orthologous genes from eukaryotic genomes (Chen *et al.*, 2006). For all genes, alignment of the smaller subset of sequences and tree building was similar to the methods described above for the reference trees.

Recruitment and placement of metagenomic reads

We analyzed 38 metagenomes from the GOS expedition (Supplementary Table 1). Only sequences from the 0.1–0.8- μ m filter size fraction were used. For a site to be included in the analysis, we required each single copy core housekeeping gene to be detected at least once to ensure sufficient *Prochlorococcus* sequence reads. In addition, site GS000a was excluded from analysis because of possible contamination in this sample with non-marine *Shewanella* and *Burkholderia* (DeLong, 2005). The unassembled metagenomic sequence reads from the GOS dataset were downloaded from the CAMERA web portal (<http://camera.calit2.net>; Sun *et al.*, 2011) on 3 February 2011. All work with the dataset was conducted locally.

For each gene (*arsR*, *arsC*, *acr3*, *arsM*, *glnA*, *rpsD* and *tyrS*), relevant metagenomic reads were recruited with *tblastn* searches using *blastall*

v 2.2.25 (Altschul *et al.*, 1997) starting with a *Prochlorococcus* MED4, MIT9313, NATL2A, SS120 and MIT9312 as well as *Synechococcus* WH8102 protein sequence as the query (Table 1). Recruited sequences with *e*-values < 1 were maintained and analyzed. Sequence reads were trimmed according to their blast alignments to ensure translation in the appropriate reading frame.

For each gene, the recruited GOS reads were aligned to the reference taxa alignment in amino acid space using PaPaRa: Parsimony-based Phylogeny-Aware Read Alignment program (Berger and Stamatakis, 2011). The metagenomic reads in the PaPaRa alignment were then assigned to nodes of the reference phylogenetic tree using EPA: Evolutionary Placement Algorithm (Berger *et al.*, 2011). All reads that were placed with appropriate *Prochlorococcus* clades in the reference trees were identified as '*Prochlorococcus*-linked metagenomic reads'. The relative abundance of genes in each metagenomic sample was calculated by normalizing the number of recruited reads to the length of the MED4 query gene and then comparing the length normalized abundance of each arsenic-related gene to the average abundance of length normalized single copy core housekeeping genes. One-way analysis of variance (ANOVA) tests were conducted to ensure that sampling sites within basins could be grouped. Mate pairs from reads identified as *Prochlorococcus*-linked were *tblastn* searched against a local *nr* database (downloaded 4/2013). In addition, the mate pairs identified as *Prochlorococcus*-linked *arsC* sequences were recruited to the AS9601 genome using the alignment program Bowtie2 (Langmead and Salzberg, 2012) and visualized with the genomic graphical viewer Tablet (Milne *et al.*, 2013).

Nutrient concentration estimation

Annual surface statistical average phosphate concentrations were collected from the World Ocean Atlas, 2009 (Garcia *et al.*, 2010), values were averaged over a one degree latitude by one degree longitude square, centered around the coordinates of the metagenomic sample sites except for station GS108a which was averaged over a two by two degree grid owing to the lack of available data (Supplementary Table 1). Minimum surface phosphate concentrations across the same spatial extents were also collected from the World Ocean Atlas, 2009.

Phosphate-binding protein ligand specificity

Indiscriminate arsenate uptake is likely mediated through the cellular phosphate uptake system which includes the high affinity uptake transporter *pstS*. To evaluate whether the specificity of the binding sites of the various copies of *pstS* among *Prochlorococcus* have changed over time, we submitted *pstS* sequences to the Phyre2 protein prediction server

(Kelley and Sternberg, 2009) as well as the 3DLi-gandSite server (Wass *et al.*, 2010).

Results

Efflux pathway arsenic detoxification genes in Prochlorococcus genomes

Prochlorococcus strains vary in their complement of arsenic detoxification genes (Table 1). Arsenate reductase, *arsC*, which reduces inorganic pentavalent arsenate to the trivalent arsenite state, is present at one copy per genome in all 12 strains for which a genome sequence is available. There is no indication of other known arsenate reductase homologs, such as *acr2* or the arsenate-reductase form of *wzb* (Bennett *et al.*, 2001) in any strain. In contrast, the efflux transporter *acr3* is differentially present among the *Prochlorococcus* strains, with copies only present in the strains MIT9301, MED4, NATL1A, NATL2A and MIT9303. There are no copies of the alternative *arsB* form of arsenite transporter among the *Prochlorococcus* strains. Thus, it appears not all *Prochlorococcus* have the genetic capacity to expel inorganic arsenite from the cell. The potential for efflux through aquaglycerolporins, which are also leaky to arsenite (Oremland and Stolz, 2003), is also unlikely as no aquaglycerolporins were identified among the *Prochlorococcus* genomes. The repressive regulator, *arsR*, is also differentially present among the strains. All strains that possess a copy of *acr3* also have the regulator *arsR*; in addition, *arsR* is also present in MIT9312, SS120 and MIT9313.

To understand the evolutionary mechanisms responsible for the variable complements of arsenic-related genes in *Prochlorococcus*, we constructed phylogenetic trees of *arsC*, *arsR* and *acr3* (Supplementary Figures 1–3) and examined their genomic context. Phylogenetic analysis of all three genes indicates a shared ancestry among the picocyanobacteria. For *acr3*, the marine picocyanobacteria form a strongly supported monophyletic group. This group is also monophyletic in *arsC* and *arsR* trees, although with less support. For all three genes, the phylogeny within the picocyanobacteria is generally congruent with ribosomal RNA phylogeny. Thus, the arsenic detoxification genes *arsC*, *acr3* and *arsR* are likely ancestral to the picocyanobacteria and the most parsimonious explanation for the differential presence of these genes among *Prochlorococcus* genomes (Table 1) is multiple independent gene loss events.

Interestingly, within the genomes of strains MED4, NATL1A and NATL2A, the genes *acr3* and *arsR* are in close genomic context to one another and to P acquisition genes associated with a genomic island (Martiny *et al.*, 2006), and show synteny with each other and with marine *Synechococcus*. However, in MIT9301 and MIT9303, only *arsR* is part of the P island and *acr3* is elsewhere. Genomic islands are defined by a preponderance of genes that have been

laterally transferred, however, they may also contain ancestral genes through genome rearrangements. In *Prochlorococcus* genes associated with genomic islands often contribute to specific environmental stress responses (Coleman *et al.*, 2006), it is not surprising to find colocation of arsenic-related genes with phosphorus stress-inducible genes on a genomic island, as the processes of phosphorus stress and arsenic uptake are intertwined. In contrast, in all *Prochlorococcus* genomes, *arsC* is located in a different region of the genome than *acr3* and *arsR*, but this location is syntenous across both *Prochlorococcus* and *Synechococcus*. The dispersed location of *arsC* in the picocyanobacteria is unusual, as in most bacteria the arsenic detoxification genes are co-located on an *ars* operon where they are regulated together (Páez-Espino *et al.*, 2009).

Identification of putative Prochlorococcus ArsM

To determine whether *Prochlorococcus* also contains the previously unknown arsenic methylation pathway, we attempted to identify an ArsM by constructing an initial phylogenetic tree of a broad array of methyltransferases belonging to COGs 500 and 2226. Within this tree, we identified a clade which includes sequences of known arsenite S-adenosylmethyltransferase (*arsM*) function (Qin *et al.*, 2006; Páez-Espino *et al.*, 2009; Yin *et al.*, 2011) as well as marine picocyanobacterial sequences. A more focused tree was constructed combining sequences from this clade with eukaryotic arsenite S-adenosylmethyltransferases (Figure 2). With the exception of *Synechococcus* WH5701, the putative *arsM* sequences of the marine picocyanobacteria form a well-supported monophyletic clade among the other *arsM* sequences. Putative *arsM* sequences were identified in the majority of *Prochlorococcus* and *Synechococcus* genomes, but were not found in *Prochlorococcus* strains NATL1A, NATL2A or SS120 (Table 1). Interestingly, a few other bacteria, including *Synechococcus* WH5701, branch within a well-supported clade consisting largely of eukaryotic sequences (from both eukaryotic algae and animals). This suggests that although *arsM* is ancestral for most of the picocyanobacteria, this gene has been subject to horizontal transfer events.

To further identify whether these *Prochlorococcus* sequences are likely functioning *arsM* orthologs, protein structure models were created for the sequence of the putative *Prochlorococcus* MED4 *arsM* as well as sequences of known arsenite S-adenosylmethyltransferase function from the freshwater cyanobacteria *Nostoc* sp. PCC 7120, *Synechocystis* sp. PCC 6803 and *Microcystis aeruginosa* NIES843 (Yin *et al.*, 2011). The protein model template *c3qnha* from the *arsM* sequence of the eukaryotic red alga *Cyanidioschyzon merolae* sp. 5508 (*Cm-arsM*) was identified as the most likely model for all the cyanobacterial sequences at greater than 90% confidence in homology (Figure 3).

Figure 2 Phylogeny of arsenite S-adenosylmethyltransferases (*arsM*). The phylogenetic tree was constructed using the maximum-likelihood program RAXML. The statistical significance of the branch pattern was estimated by conducting a 100 bootstrap replications of the original amino acid alignment; bootstraps ≥ 50 shown. The related SAM-dependent mycolic acid cyclopropane synthetase (*cmaS*) gene was used as an outgroup. Bold sequences represent sequences of genes with confirmed ArsM function (Rosen, 2002; Qin *et al.*, 2006; Yin *et al.*, 2011).

Cysteine residues integral to the binding of arsenite to ArsM protein at positions 72, 174 and 224 in *CmArsM* (Ajees *et al.*, 2012) are conserved among all the cyanobacterial sequences, including the putative *Prochlorococcus* MED4 *arsM*. Overall secondary structure and SAM-binding motifs are also conserved. The similarity in these key catalytic residues among the cyanobacteria and the red algae indicate likely conservation of function.

Frequency of arsenic-related genes in wild *Prochlorococcus* populations

To understand the selective pressures on *Prochlorococcus* arsenic-related genes in the environment, we examined the frequency of these genes in global *Prochlorococcus* populations. The Global Ocean Survey sampling sites used for this analysis were predominately from the Sargasso Sea, Caribbean Sea, Eastern Pacific Tropical Upwelling Region and the

Figure 3 Amino acid pairwise alignment of cyanobacterial sequences to the red algae *Cyanidioschyzon merolae* sp. 5508 ArsM. Predicted secondary structures generated by the fully automated protein homology/analogy recognition engine Phyre2 (Kelley and Sternberg, 2009) are depicted below the amino acids. Red arrows above amino acid alignment indicate conserved residues identified to be integral for catalysis of the arsenic methylation reaction in *C. merolae* (Ajees *et al.*, 2012). Red boxes indicate conserved motifs of regions involved in SAM binding to the protein. Purple loops in predicted secondary structure symbolize alpha helices and teal arrows symbolize predicted beta sheets.

Figure 4 Boxplot of the statistical average annual phosphate concentration at the surface for the GOS metagenome sites analyzed in this study according to ocean basin location. Outliers are represented by stars.

Indian Ocean (Supplementary Table 1). The three oceanic basins sampled captured three significantly different phosphate regimes (Figure 4) (ANOVA P -value < 0.000). The Atlantic sites have the lowest annual average phosphate conditions ($0.08 \mu\text{mol l}^{-1}$), the Indian basin sites were higher at $0.18 \mu\text{mol l}^{-1}$. The Pacific sites had the highest average phosphate concentrations ($0.49 \mu\text{mol l}^{-1}$) and also the greatest variability among sites. The same pattern of phosphate regimes was identified when comparing phosphate minima, with the Atlantic experiencing

the most extreme phosphate minima, the Indian at moderate levels and the Pacific sites experiencing the highest minimum phosphate conditions (ANOVA P -value < 0.000). Thus, this dataset effectively captured *Prochlorococcus* populations exposed to low, moderate and relatively high phosphate conditions.

The presence of arsenic efflux detoxification genes compared with single copy core genes varies among global *Prochlorococcus* populations (Figure 5). The average occurrence of the single copy core genes *glnA*, *rpsD* and *tyrS* were used as a proxy for the total number of *Prochlorococcus* genomes sampled. These genes occur at a rate of one copy per genome among cultured *Prochlorococcus* (Kettler *et al.*, 2007) and their occurrence in metagenomes did not significantly vary between basins (ANOVA P -value for *glnA*, *rpsD* and *tyrS*: 0.112, 0.056 and 0.105, respectively). The majority of environmental reads for all genes were placed within the high light *Prochlorococcus* clades. This is not surprising as most of these sites were sampled from 5-m depth or shallower. Among the global *Prochlorococcus* populations, the *arsR* regulator occurs at less than one copy per genome on average. The *acr3* arsenite efflux transporter also occurs in less than one copy per genome, and was not detected at all in some populations. In contrast, the *arsC* arsenate reductase appears to be present at greater than one copy per genome on average in global *Prochlorococcus* populations. The putative *arsM* arsenite *S*-adenosylmethyltransferase is present at an average of one

copy per genome among global *Prochlorococcus* populations (Figure 5a)

We observed basin scale differences in the relative occurrence of some arsenic detoxification genes (Figure 5, Table 2). In the phosphate-poor Atlantic sites, the *arsR* regulator appears at about one copy per *Prochlorococcus* genome (Figure 5b), but it is significantly less frequent in Indian and Pacific populations (ANOVA P -value <0.000). *acr3*, the component of the efflux pathway responsible for expulsion of arsenite from the cell, is present in Atlantic populations at less than one copy per *Prochlorococcus* genome, still significantly more

(ANOVA P -value <0.000) than in Indian and Pacific basin populations, where it was not detected in many samples. In contrast, the *arsC* arsenate reductase gene occurs in two copies per *Prochlorococcus* genome in the Atlantic and Indian oceans, but this apparent duplication is not present in Pacific populations. The *arsM* methyltransferase occurs at about one copy per genome on average in all global *Prochlorococcus* populations. Thus, *Prochlorococcus* populations in the Atlantic are capable of arsenic methylation and a portion of the population maintains the genomic potential for arsenite efflux, whereas the *Prochlorococcus* populations in the Indian and Pacific basins contain the methylation pathway but have lost the capability for arsenite efflux.

Discussion

Arsenic is the most abundant of environmental toxins with toxicity rooted in its chemical similarity to phosphorus. We suggest that organisms can be susceptible to arsenic toxicity, even at relatively low absolute arsenic concentrations, if phosphorus concentrations are also very low. The marine cyanobacteria *Prochlorococcus* thrives in one such low P environment, the tropical and subtropical oligotrophic oceans. *Prochlorococcus* strains possess differing genomic potentials for arsenic detoxification and the presence/absence pattern of these arsenic-related genes is not congruent with evolutionary relationships based on rRNA genes. Our phylogenetic reconstructions found that the marine picocyanobacteria consistently form a monophyletic clade (Figure 2 and Supplementary Figures 1–3) suggesting that multiple independent gene loss events are responsible for the observed pattern (Table 1). We suggest that the *acr3* arsenite efflux transporter and the *arsR* regulator are being lost where they are no longer needed. Notably, the strains lacking both of these genes (MIT9515, MIT9215, MIT9211 and AS9601) were isolated from either the Equatorial Pacific or the Arabian Sea (Table 1; Rocap *et al.*, 2002) where P is not typically limiting. They are also the strains observed to have fewest P acquisition genes (Martiny *et al.*, 2006) indicating

Figure 5 Boxplots of the relative *Prochlorococcus*-linked arsenic detoxification gene abundance within the GOS metagenomes of the Atlantic, Pacific and Indian basins. The shaded region represents one copy of a gene per genome; the spread of this shading around 1 represents the error associated with the estimation of *Prochlorococcus* genomes within the basin using single copy core gene abundance. (a) Average across all basins, (b) Atlantic sites, (c) Indian sites and (d) Pacific sites. Outliers are represented by stars.

Table 2 Statistical analysis of relative abundance of arsenic detoxification genes in the various ocean basins

	ANOVA P -value	Partial Eta Squared	Tukey test		
			Atlantic vs Indian	Atlantic vs Pacific	Indian vs Pacific
ArsR	0.002*	0.306	0.007*	0.002*	0.737
ArsC	0.006*	0.252	0.987	0.044	0.008*
ACR3	0.000*	0.641	0.000*	0.000*	0.808
ArsM	0.355	0.057	N/A	N/A	N/A
[PO ₄]	0.000*	0.679	0.165	0.000*	0.000*

Abbreviation: ANOVA, analysis of variance. P -values <0.05 in bold; P -values <0.01 with *. A one-way ANOVA was conducted on each gene set, and significantly different gene sets (P -value <0.05) were analyzed for pairwise differences between basins using Tukey's *post hoc* test.

that the relaxation of pressures induced by low P has allowed the loss of these genes.

The relative occurrences of arsenic-related genes in *Prochlorococcus* vary in wild populations as well, where populations from low phosphate regimes maintain a greater genomic potential for arsenic detoxification. The arsenic efflux transporter, and therefore the complete efflux detoxification pathway, is only significantly present in *Prochlorococcus* populations in the Atlantic Ocean, where annual average phosphate concentrations in the sites sampled are $0.08 \mu\text{mol l}^{-1}$. These results are consistent with previous work which found an enrichment of *Prochlorococcus*-linked *acr3* genes at the Bermuda Atlantic Time Series station compared with the Hawaii Ocean Time Series in the North Pacific (Coleman and Chisholm, 2010). *ArsR* may also be in the process of being lost from some *Prochlorococcus* populations, as it occurs at less than one copy per genome in the Indian and Pacific basins. We estimated a $\text{PO}_4\text{:AsO}_4$ ratio as 5:1, 11:1 and 30:1 for the Atlantic, Indian and Pacific sites, respectively. Because data on arsenate concentrations are sparse, this calculation used an arsenate concentration of 15 nmol l^{-1} (Cutter *et al.*, 2001; Ellwood and Maher, 2002; Cutter and Cutter, 2006) combined with site-specific annual average phosphate concentrations. Thus, the arsenic efflux detoxification pathway is not present in the majority of the *Prochlorococcus* populations globally, and may be a secondary detoxification pathway that is present only where risk of indiscriminate arsenate uptake is very high, which we suggest occurs when $\text{PO}_4\text{:AsO}_4$ ratios approach 5:1 or lower. In contrast, the newly identified methylation strategy appears to be a more universal strategy for responding to arsenic in the environment.

Phosphate concentrations low enough to result in P-limitation may also result in enhanced arsenic uptake. The high affinity periplasmic phosphate-binding protein PstS (Figure 1) has an important role in the uptake of arsenate into cells. *PstS* expression significantly increases under both phosphate limitation and starvation (Martiny *et al.*, 2006; Reistetter *et al.*, 2013) resulting in an increase in phosphate uptake rates compared with P-replete growth conditions (Krumhardt *et al.*, 2013). Thus, in a low $\text{PO}_4\text{:AsO}_4$ environment, this phosphate stress response, which increases phosphate scavenging, may result in an increase in arsenic uptake. However, it is worth noting that an additional strategy for cellular arsenic defense is the prevention of indiscriminate uptake of arsenate in the first place. *Prochlorococcus* MIT9313, which lacks the *acr3* gene, possesses two copies of *pstS*, only one of which is expressed in response to phosphate stress (Martiny *et al.*, 2006). Recent work on the two periplasmic phosphate-binding proteins in the extremely arsenic-tolerant *Halomonas* strain GFAJ-1 from Mono Lake has shown that one of the paralogs can discriminate phosphate from arsenate

4500-fold, compared with 500-fold discrimination of the other copy (Elias *et al.*, 2012). We explored this possibility using protein secondary structure prediction combined with binding site specificity modeling which suggests that the various *Prochlorococcus* PstS-binding proteins may vary in their ligand-binding specificities. Thus, it is possible that some PstS proteins may have greater capability to discriminate arsenate from phosphate than others, however, additional experiments such as competitive uptake assays would be required to verify this.

Arsenate reductase, *arsC*, is required by both the efflux pathway and the methylation pathway and is present in a single copy in all isolate *Prochlorococcus* strains. Strikingly, it appears to be duplicated in environmental *Prochlorococcus* populations in the Atlantic and the Indian basins, but not in the Pacific, where it occurs at an average of one copy per genome. The duplicated copies of *arsC* in these populations could confer amplified or differentially regulated expression of arsenate reductase, or may be paralogous genes of another function, although currently there are no known paralogs of this evolutionary form of *arsC* (Mukhopadhyay *et al.*, 2002). Duplicate copies of an arsenate reductase have been identified in other organisms, including the freshwater cyanobacterium *Synechocystis* PCC 6803, where they are often found on plasmids (Kaneko *et al.*, 2003; Páez-Espino *et al.*, 2009). It is also possible the *arsCs* we detected are on cyanophage genomes. Although no *arsC*-containing cyanophage genomes have been observed, *Prochlorococcus* phosphate-stress-inducible genes, such as *pstS* and *phoH*, are commonly found on cyanophages (Sullivan *et al.*, 2005), and there is evidence that environmental phosphorus conditions are a main selective agent on cyanophage genomes (Kelly *et al.*, 2013). It is also possible that the additional copies represent a very recent horizontal transfer from *Prochlorococcus* to another organism. However, the associated mate pairs from *arsC* reads were also identified as *Prochlorococcus*-like and the majority recruited to the same localized region of a *Prochlorococcus* genome, thus, at present, the source and function of the additional copies of *arsC* is unclear.

Prior to this work, the only known arsenic detoxification pathway in *Prochlorococcus* was the efflux pathway which relies on the ACR3 arsenite efflux transporter (Scanlan *et al.*, 2009). Here, we identified a putative arsenite S-adenosylmethyltransferase, *arsM*, which is present in most sequenced *Prochlorococcus* strains and appears to be maintained at about one copy per *Prochlorococcus* genome globally. The presence of *arsM* indicates that *Prochlorococcus* likely has the capacity to methylate inorganic arsenicals into less toxic organic forms such as monomethylarsonic acid, dimethylarsonic acid or the gas TMAO(g) and potentially to biosynthesize additional organoarsenical compounds such as arsenolipids and arsenosugars, which are commonly found in eukaryotic algae (Dembitsky and Levitsky, 2004). The widespread

occurrence of *arsM* in global *Prochlorococcus* populations suggests that the methylation pathway may be part of a general metabolic strategy rather than serving solely in arsenic detoxification. For example, *Prochlorococcus* uses sulfolipids in place of phospholipids in the membrane, thereby reducing the cellular phosphorus demand (Van Mooy *et al.*, 2006) and it is possible that synthesis of arsenolipids allows further reduction of cellular P quotas. For example, the eukaryotic algae *Chlorella* methylates inorganic arsenicals and produces polar arsenolipids (Lunde, 1973; Dembitsky and Levitsky, 2004). Arsenosugars are the predominate form of arsenic found in algae, and the freshwater cyanobacteria *Synechocystis* sp. PCC 6803 and *Nostoc* sp. PCC 7120 are also capable of producing arsenosugars (Miyashita *et al.*, 2012). Recent work suggests that human ingestion of arsenosugars and arsenolipids might be harmful with potential for release of toxic metabolites with arsenolipids posing the potential for toxicity at levels comparable with inorganic arsenite (Harrington *et al.*, 2008; Raml *et al.*, 2009; Newcombe *et al.*, 2010; Feldmann and Krupp, 2011; Leffers *et al.*, 2013a,b; Meyer *et al.*, 2014). Further laboratory work is needed to verify the functionality of *arsM* and to determine to what extent *Prochlorococcus* is capable of methylating arsenicals and what organic compounds are synthesized.

The ratio of phosphate to arsenate appears to exert influence on the biological conversion of inorganic arsenate to other forms, as the cycling of these two elements are inextricably linked. Along an open ocean transect that covered varying phosphate regimes, when the $\text{PO}_4\text{:AsO}_4$ ratio was less than 1, the total arsenic pool consisted of a greater proportion of reduced and methylated arsenicals compared with the relatively phosphate-rich region, with an inorganic $\text{PO}_4\text{:AsO}_4$ ratio of 100:1 (Cutter and Cutter, 2006). In the North Atlantic, both methylated arsenicals and arsenite detoxification products have been identified and the spatial distribution of arsenite closely corresponded with phosphate availability and can be used in conjunction with measures of alkaline phosphatase to identify regions of phosphorus limitation (Wurl *et al.*, 2013). There is also evidence that the diazotrophic cyanobacterial communities in these phosphate-poor regions are responding to arsenic toxicity. The expression of *arsC*, *arsA* and *arsB* were detected in a metatranscriptome of a *Trichodesmium* bloom, suggesting microbial response to arsenic toxicity associated with nitrogen fixation (Hewson *et al.*, 2009). Additionally, expression of the *Crocospaera watsonii* WH8105 arsenite efflux transporters increases in laboratory cultures when arsenate is present and the cells are phosphate-stressed. In the oligotrophic Sargasso Sea, with a DIP concentration $<0.01 \mu\text{mol l}^{-1}$, a transcript for a *C. watsonii* WH8105 arsenic efflux transporter was successfully identified indicating an *in situ* response to arsenic toxicity (Dyhrman and Haley, 2011).

The sheer abundance of *Prochlorococcus*, which can grow at densities greater than 7×10^5 cells ml^{-1} (Campbell *et al.*, 1998), makes it a key player in biogeochemical cycling of arsenic in the open ocean. As warming associated with climate change causes more intense and persistent stratification of the upper water column, phosphate scarcity and corresponding arsenic exposure will be exacerbated resulting in an increased cycling of arsenicals mediated by *Prochlorococcus*. The two detoxification pathways have both different end products and intermediary substrates with a broad range of associated toxicities. The previously known but less widespread efflux pathway expels arsenite, which is more toxic than arsenate, from the cell back into the environment where it can either be oxidized back into arsenate in a matter of hours to days (Cutter, 1992) or taken up by eukaryotic organisms, such as algae and fish, *via* diffusion, hexose permease transporters and aquaporins (Ventura-Lima *et al.*, 2011). The methylation pathway is more effective at sequestering the toxic effects of arsenic, as the detoxified products monomethylarsonic acid and dimethylarsinic acid have a residence time on the order of years rather than days (Cutter and Cutter, 2006), and TMAO(g) may remove arsenic altogether from the marine system. However, the methylation and storage of organoarsenicals by *Prochlorococcus* may result in bioaccumulation of complexed organoarsenicals in upper trophic levels (Dembitsky and Levitsky, 2004). Prior to the identification of *arsM*, active arsenic detoxification by *Prochlorococcus* in the environment would presumably only have contributed to the transformation of inorganic arsenate to arsenite, which would then be rapidly oxidized back to arsenate. In addition, this interaction would only occur in the most phosphate-stressed regions of the ocean. Our identification of the *arsM* gene which is widespread among global *Prochlorococcus* populations suggests *Prochlorococcus* has a more complicated impact on global arsenic cycling through the production of organoarsenicals.

Conclusions

Our analysis suggests that *Prochlorococcus* utilizes arsenic detoxification mechanisms in oceanic regions of phosphorus scarcity where the $\text{PO}_4\text{:AsO}_4$ ratios are low. There are two detoxification pathways in *Prochlorococcus*, the efflux pathway (Scanlan *et al.*, 2009) and the putative arsenic methylation pathway identified by this work. The methylation pathway is nearly ubiquitous in global *Prochlorococcus* populations and likely confers some basal arsenic resistance; however, the efflux pathway is only maintained in populations which experience extreme phosphorus scarcity and have a greater possibility of arsenic uptake into the cell due to reduced $\text{PO}_4\text{:AsO}_4$ ratios in the environment. Our analysis also suggests that the ability to detoxify

arsenic is an ancestral state in marine picocyanobacteria. It is vital to understand the products of the different arsenic biochemical pathways, their global occurrence and the rates of detoxification, as the chemical transformations of arsenic by these two pathways are vastly different and have ecological implications for arsenic biogeochemical cycling.

Conflict of Interest

The authors declare no conflict of interest.

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