

ORIGINAL ARTICLE

Physiology and evolution of nitrate acquisition in *Prochlorococcus*

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***Prochlorococcus* is the numerically dominant phototroph in the oligotrophic subtropical ocean and carries out a significant fraction of marine primary productivity. Although field studies have provided evidence for nitrate uptake by *Prochlorococcus*, little is known about this trait because axenic cultures capable of growth on nitrate have not been available. Additionally, all previously sequenced genomes lacked the genes necessary for nitrate assimilation. Here we introduce three *Prochlorococcus* strains capable of growth on nitrate and analyze their physiology and genome architecture. We show that the growth of high-light (HL) adapted strains on nitrate is ~17% slower than their growth on ammonium. By analyzing 41 *Prochlorococcus* genomes, we find that genes for nitrate assimilation have been gained multiple times during the evolution of this group, and can be found in at least three lineages. In low-light adapted strains, nitrate assimilation genes are located in the same genomic context as in marine *Synechococcus*. These genes are located elsewhere in HL adapted strains and may often exist as a stable genetic acquisition as suggested by the striking degree of similarity in the order, phylogeny and location of these genes in one HL adapted strain and a consensus assembly of environmental *Prochlorococcus* metagenome sequences. In another HL adapted strain, nitrate utilization genes may have been independently acquired as indicated by adjacent phage mobility elements; these genes are also duplicated with each copy detected in separate genomic islands. These results provide direct evidence for nitrate utilization by *Prochlorococcus* and illuminate the complex evolutionary history of this trait.**

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Introduction

The unicellular cyanobacterium *Prochlorococcus* is the smallest known free-living oxygenic phototroph (Chisholm *et al.*, 1992; Partensky *et al.*, 1999; Coleman and Chisholm, 2007; Partensky and Garczarek, 2010). It is numerically dominant in the tropical and subtropical regions of the world's oceans and responsible for 5–10% of marine

primary productivity (Campbell *et al.*, 1994; Partensky *et al.*, 1999; Buitenhuis *et al.*, 2012; Flombaum *et al.*, 2013). *Prochlorococcus* has undergone a process of genome reduction following divergence from its closest relatives, the marine *Synechococcus* (Rocap *et al.*, 2002; Kettler *et al.*, 2007). These streamlined genomes are often considered an adaptation to the oligotrophic environments they occupy (Dufresne *et al.*, 2003; Rocap *et al.*, 2003). Even though individual genomes are small, the collective of all *Prochlorococcus* cells possesses a vast reservoir of genetic and physiological diversity (Kettler *et al.*, 2007). *Prochlorococcus* is composed of a polyphyletic group of low-light (LL) adapted clades (LLI–LLVI and NC1), and a more recently diverged monophyletic group of high-light

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(HL) adapted clades (HLI–HLVI) (Moore *et al.*, 1998; Moore and Chisholm, 1999; Rocap *et al.*, 2002; Martiny *et al.*, 2009c; Lavin *et al.*, 2010; Shi *et al.*, 2011; Huang *et al.*, 2012; Malmstrom *et al.*, 2013). Some of these clades are known to be differentially distributed along gradients of light intensity, temperature and nutrient concentrations (Bouman *et al.*, 2006; Johnson *et al.*, 2006; Zinser *et al.*, 2006; Zwirgmaier *et al.*, 2007, 2008; Malmstrom *et al.*, 2010, 2013).

Nitrogen availability often limits primary productivity in marine systems (Tyrrell, 1999), and organisms have evolved diverse mechanisms for uptake of various chemical forms of nitrogen. Nitrate is one of the more abundant sources of inorganic nitrogen available to phytoplankton (Gruber, 2008), and the majority of cyanobacteria possess pathways for the uptake and assimilation of nitrate (Herrero *et al.*, 2001; García-Fernández *et al.*, 2004; Ohashi *et al.*, 2011). Early reports on the vertical distributions of *Prochlorococcus* noted a subsurface maximum in abundance at the base of the euphotic zone, suggesting that *Prochlorococcus* was sensitive to nitrogen depletion and might be assimilating nitrate supplied from deep waters (Olson *et al.*, 1990; Vaultot and Partensky, 1992). Therefore, it was surprising that nearly all isolates of *Prochlorococcus* could not use nitrate and lacked the genes required for this function (Moore *et al.*, 2002; Coleman and Chisholm, 2007; Kettler *et al.*, 2007), even though most isolates of *Synechococcus* are capable of using nitrate (Fuller *et al.*, 2003; Ahlgren and Rocap, 2006). Only a single *Prochlorococcus* culture, PAC1 isolated in 1992, was reported to utilize nitrate (Williams *et al.*, 1999), but because of the presence of other bacteria in that culture, direct nitrate uptake by *Prochlorococcus* could not be conclusively demonstrated.

Several pieces of evidence indicated that nitrate assimilation was a more common trait within *Prochlorococcus* populations than previously thought. Field experiments demonstrated the uptake of isotopically labeled nitrate by *Prochlorococcus* cells in the Sargasso Sea (Casey *et al.*, 2007), and nitrate assimilation genes were found to be associated with uncultivated *Prochlorococcus* genomes from many regions of the subtropical oceans (Martiny *et al.*, 2009b). A scaffold assembled from metagenomic data from the Global Ocean Sampling (GOS) expedition indicated that all the genes required for nitrate assimilation were colocalized in a specific region of the genomes of HL adapted *Prochlorococcus*. The metagenomic data primarily identified nitrate utilization genes in the HLII clade of *Prochlorococcus* as sequences from this clade comprised the majority of *Prochlorococcus*-like sequences in the GOS data set (Rusch *et al.*, 2007).

These past observations raised two important questions about nitrate assimilation in *Prochlorococcus*. (1) Can axenic strains grow on nitrate as the

sole nitrogen source? (2) What is the evolutionary history of nitrate assimilation genes in this group? To address these questions, we isolated and sequenced *Prochlorococcus* strains capable of nitrate assimilation and examined their growth on different nitrogen sources. We then used comparative genomics to better understand how this trait had evolved in *Prochlorococcus*.

Materials and methods

Strains and enrichments

Five strains of *Prochlorococcus* (SB, MIT0604, PAC1, MIT9301 and MED4), one strain of *Synechococcus* (WH8102) and two *Prochlorococcus* enrichment cultures (P0902-H212 and P0903-H212) were used in this study. MIT9301, MED4 and WH8102 have previously been rendered axenic (free of heterotrophic contaminants). All axenic cultures were routinely assessed for purity by confirming a lack of turbidity after inoculation into a panel of purity test broths: ProAC (Morris *et al.*, 2008), MPTB (Saito *et al.*, 2002) and ProMM (Pro99 medium (Moore *et al.*, 2007) supplemented with $1 \times$ Va vitamin mix (Waterbury and Willey, 1988) and 0.05% w/v each of pyruvate, acetate, lactate and glycerol). ProMM is a modified version of the PLAG medium (Morris *et al.*, 2008), but uses 100% sea water as the base.

PAC1 was enriched from sea water collected from the deep chlorophyll maximum in the North Pacific Ocean at Station ALOHA (22.75°N, 158°W) on Hawai'i Ocean Time-series (HOT) cruise 36. Sea water was passed through a 0.6 μ m Nucleopore filter twice, and the filtrate was serially diluted into K/10 medium (Chisholm *et al.*, 1992), but with the following modifications for final nutrient concentrations: 5 μ M urea, 5 μ M ammonium and 1 μ M β -glycerophosphate replacing inorganic phosphate, 0.01 μ M Na₂MoO₄ and 0.05 μ M NiCl₂. MIT0604 was derived from an enrichment culture initiated with Pro2 nutrient additions (Moore *et al.*, 2007) to sea water obtained at Station ALOHA on HOT cruise 181, but with all nitrogen sources replaced by 0.217 mM sodium nitrate. The P0902-H212 and P0903-H212 enrichments were initiated with Pro2 nutrient additions (Moore *et al.*, 2007) to sea water obtained from Station ALOHA on HOT cruise 212, but with all nitrogen sources replaced by 0.05 mM sodium nitrate.

Purification of Prochlorococcus strains

SB and MIT0604 were rendered axenic in this study using a modified dilution to extinction method. *Prochlorococcus* from exponential phase cultures were enumerated using an Influx Cell Sorter (BD Biosciences, San Jose CA, USA) or a FACSCalibur flow cytometer (BD Biosciences) as previously described (Olson *et al.*, 1985; Cavender-Bares *et al.*, 1999). Cultures consisting of >80% *Prochlorococcus* cells were serially diluted into multiple multiwell

plates at final concentrations of 1–10 cells per well in at least 200 μl of ProMM medium. Axenic *Prochlorococcus* do not grow from such low cell densities in Pro99 medium without ‘helper’ heterotrophic bacteria (Morris *et al.*, 2008, 2011); however, they do grow when diluted into ProMM. The main ingredient in ProMM that promotes the growth of cells from low densities is pyruvate, and we suspect that in this context pyruvate serves as a potent hydrogen peroxide scavenger (Giandomenico *et al.*, 1997). Wells contaminated with heterotrophic bacteria were identified by the appearance of turbidity. The multiwell plates were monitored by eye and by fluorometry using a Synergy HT Microplate Reader (BioTek, Winooski, VT, USA), and nonturbid wells were monitored by flow cytometry using a FACSCalibur flow cytometer. Wells that appeared green or had *Prochlorococcus* cells as determined by flow cytometry were immediately transferred to Pro99 medium directly, or into fresh ProMM medium until consistent growth was observed, at which point the cultures were introduced back into Pro99 medium. Cultures were examined for heterotrophic bacteria contaminants by flow cytometry and by inoculation into the panel of purity test broths as described above.

PCR screen for the nitrate reductase gene

Based on an alignment of GOS reads coding for the *Prochlorococcus narB* sequence (Martiny *et al.*, 2009b), degenerate primers 30narB175f (5'-TGYGTDAAAGGMGCAACAGTNTG-3') and 30narB574r (5'-GACAYTCWGCBGATTTWGTGCC-3') were designed to specifically amplify the *narB* gene from HLII clade *Prochlorococcus*, and degenerate primers 40narB1447f (5'-TATTGYCCAGCWTTTYMGDCCDTG-3') and 40narB1766r (5'-AKAGGWTGYTTWGTTRTARAAYTG-3') were designed to specifically amplify the *narB* gene from LLI clade *Prochlorococcus*. PCR used annealing temperatures of 52.5 °C for the HLII *narB* sequence and 56 °C for the LLI *narB* sequence. Reactions contained 1 \times PCR buffer, 2.5 mM MgCl_2 , 0.2 mM each of dATP, dTTP, dCTP and dGTP, 0.2 μM of each primer, 1 unit of Platinum Taq DNA polymerase (Life Technologies, Grand Island, NY, USA) and 1 ng of genomic DNA prepared from *Prochlorococcus* cultures in the MIT Cyanobacteria Culture Collection (Chisholm Laboratory, MIT, Cambridge, MA, USA). DNA from *Synechococcus* WH8102, which contains a *narB* gene, was used as a negative control. Reactions were cycled 30 times at 94 °C for 15 s, the primer-specific annealing temperature for 15 s and 72 °C for 60 s. PCR products with the expected size were sequenced at the Dana-Farber/Harvard Cancer Center DNA Resource Core (Boston, MA, USA) to confirm amplification of the *narB* gene.

Growth in the presence of alternative nitrogen sources

Axenic *Prochlorococcus* strains SB, MIT0604, MIT9301 and MED4, and axenic *Synechococcus*

strain WH8102 were acclimated to Pro99 medium (Moore *et al.*, 2007) prepared with sea water from the South Pacific Subtropical Gyre and grown at 24 °C and 50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ continuous illumination for at least 10 generations or until growth rates were similar between successive transfers. Bulk culture fluorescence was measured as a proxy for biomass using a 10-AU fluorometer (Turner Designs, Sunnyvale, CA, USA). Triplicate cultures of each strain were initiated in Pro99 that contained 0.8 mM ammonium chloride. Once cultures had reached mid-exponential phase, they were transferred into Pro99 medium containing 0.8 mM ammonium chloride, 0.8 mM sodium nitrate, 0.8 mM sodium cyanate or no nitrogen additions as a control to monitor utilization of carry-over ammonium. Cultures were successively transferred at mid-exponential phase until growth in the cultures lacking nitrogen additions had arrested because of nitrogen limitation. Specific growth rates were estimated from the log-linear portion of the growth curve for the final transfer. Two tailed homoscedastic *t*-tests were conducted in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) in order to evaluate the likelihood of significantly different growth rates in each strain for each pair of nitrogen sources and for strains grown on the same nitrogen source.

Genome data

A total of 41 *Prochlorococcus* and 15 *Synechococcus* genomes (Biller *et al.*, 2014) that include the genomes of the nitrate assimilating strains SB, MIT0604 and PAC1 were used in this study. Sequence data were also obtained for the P0902-H212 and P0903-H212 enrichment cultures as described in the Supplementary Methods. These enrichment assemblies had total sequence lengths approximately twice the size of previously sequenced *Prochlorococcus* genomes, suggesting the presence of at least two unique strains dominating each enrichment. Binning contigs based on average sequencing coverage yielded a subset of highly covered contigs in each assembly with a total sequence length similar to that of previously sequenced *Prochlorococcus* genomes. In the highly covered subsets for each assembly, the complete set of nitrate assimilation genes were only found on a single contig. For the purpose of this study, only these contigs were relevant and entered into our analysis.

All sequence data were annotated using the RAST server (Aziz *et al.*, 2008) with FIGfam release 49 in order to facilitate comparison between genomes by ensuring a uniform methodology for gene calling and functional annotation. Clusters of orthologous groups of proteins (COGs) were identified as previously described (Kelly *et al.*, 2012). These clusters are included in the ‘V4’ CyCOGs on the ProPortal website (<http://proportal.mit.edu>) (Kelly *et al.*, 2012; Biller *et al.*, 2014).

Genome phylogeny

We translated 537 single-copy core genes to amino acid sequences, aligned each gene individually in protein space using ClustalW (Larkin *et al.*, 2007), and then back-translated the sequences using TranslatorX (Abascal *et al.*, 2010). Using the principle previously described (Kettler *et al.*, 2007), we randomly concatenated 100 of these aligned genes and built maximum likelihood and neighbor-joining phylogenies using PHYLIP v3.69 (Felsenstein, 2005). We repeated the random concatenation and tree generation 100 times.

Estimation of gene gain and loss

Using a maximum parsimony approach (Mirkin *et al.*, 2003), the patterns of gene gain and loss were mapped onto the topology of the maximum likelihood nucleotide tree using WH5701 as an outgroup. Utilizing 13 590 non-core single-copy COGs, we reconstructed ancestral character states of gene absence and presence on our guide tree and minimized the cost of gains and losses given a gene gain equal to twice a gene loss. We used the program DendroPy to implement the tree traversal portion of the algorithm (Sukumaran and Holder, 2010).

Phylogenies of genes involved in the transport and reduction of nitrate and nitrite

COGs corresponding to the *nirA*, *narB*, *focA* and *napA* genes were aligned in protein space using ClustalW. Phylogenetic trees were estimated with PHYLIP v3.69 (Felsenstein, 2005) using the programs SEQBOOT, PROTDIST with the Jones–Taylor–Thornton matrix and a constant rate of variability among sites and NEIGHBOR on the aligned amino acid sequences with *Synechococcus* WH5701 used as an outgroup for *nirA* and *narB* and *Synechococcus* CB0101 used as an outgroup for *focA* and *napA*. We included GOS consensus sequences: GOS *nirA*, GOS *narB* and GOS *napA* (Martiny *et al.*, 2009b).

Results and discussion

Isolates of *Prochlorococcus* are capable of nitrate assimilation

To identify possible cultures capable of nitrate assimilation, we screened existing *Prochlorococcus* cultures for the assimilatory nitrate reductase gene, *narB*, using PCR. We found that the LL adapted PAC1 strain (Penno *et al.*, 2000) and the HL adapted SB strain (Shimada *et al.*, 1995) each contained the gene. In search of additional strains capable of utilizing nitrate, we performed selective enrichments from sea water obtained from the subtropical North Pacific Ocean using nitrate as the sole added nitrogen source. This yielded one HL adapted strain (*Prochlorococcus* MIT0604) and two mixed *Prochlorococcus* cultures (P0902-H212 and P0903-H212) with the *narB* gene (Table 1).

We then rendered SB and MIT0604 axenic and examined their growth in the presence of nitrate or ammonium. As hypothesized, both SB and MIT0604 can grow on nitrate as the sole source of nitrogen, but with a significant reduction in growth rate (18% and 17%, respectively), compared with growth on ammonium (Figure 1 and Supplementary Figure S1). Although the slower growth on nitrate could be explained by the greater amount of reducing power required to assimilate more oxidized N sources (García-Fernández *et al.*, 2004), we assume that these cultures were growing at saturating light intensities based on previous measurements of light saturating irradiances for the growth of *Prochlorococcus* (Moore and Chisholm, 1999); thus energy supply and reducing power were likely not limiting. Furthermore, recent work has shown that the growth rates and chemical composition of some marine cyanobacteria are not directly related to the oxidation state of the cells' N source (Collier *et al.*, 2012). Under light-limiting conditions, for example, the growth rate and chemical composition of *Synechococcus* grown on ammonium was the same as that on nitrate; however, under light-saturating conditions, cells grown on nitrate had a higher carbon-to-nitrogen ratio (Collier *et al.*, 2012). This perhaps suggests a bottleneck in the uptake and conversion of nitrate compared with ammonium when energy is sufficient (Collier *et al.*, 2012), and may explain the

Table 1 *Prochlorococcus* strains and enrichments capable of growth in the presence of nitrate as the sole nitrogen source

Name	Clade	Axenic	Isolation depth (m)	Isolation coordinates	Region	Isolation date	Assembly size (bp)	Contigs	% GC	Genbank accession	Reference
<i>Unialgal cultures (complete genome sequences)</i>											
SB	HL II	Yes	40	35°N, 138.3°E	Suruga Bay, Japan	October 1992	1 668 514	3	31.5	JNAS00000000	Shimada <i>et al.</i> (1995); Biller <i>et al.</i> (2014)
MIT0604	HL II	Yes	175	22.75°N, 158°W	North Pacific	May 2006	1 780 061	1	31.2	CP007753	This study
PAC1	LL I	No	100	22.75°N, 158°W	North Pacific	April 1992	1 825 493	15	35.1	JNAX00000000	Penno <i>et al.</i> (2000); Biller <i>et al.</i> (2014)
<i>Mixed enrichments (partial genome assemblies)</i>											
P0902-H212	LL I	No	175	22.75°N, 158°W	North Pacific	July 2009	501 825	1	35.4	KJ947870	This study
P0903-H212	LL I	No	200	22.75°N, 158°W	North Pacific	July 2009	291 739	1	35.2	KJ947871	This study

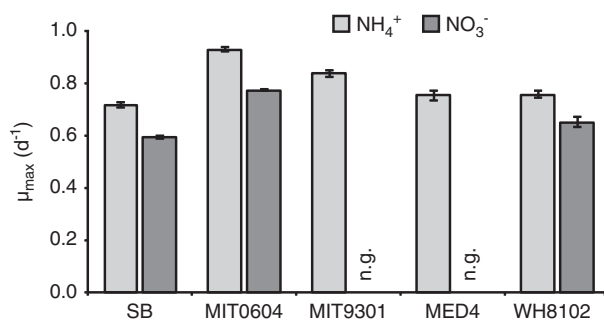


Figure 1 Maximum specific growth rates (μ_{max}) of *Prochlorococcus* strains SB, MIT0604, MIT9301, MED4 and *Synechococcus* WH8102 in the presence of ammonium or nitrate. Values represent the mean and s.d. of three biological replicates. Growth rate differences for each strain grown on ammonium compared with nitrate as well as growth rate differences between strains on the same nitrogen source were significant ($P < 0.05$) in a two-tailed homoscedastic *t*-test; n.g., no growth.

slower growth of *Prochlorococcus* on nitrate compared with ammonium.

In the early days of research on *Prochlorococcus*, the absence of cultures known to utilize nitrate resulted in a distorted view of the role of *Prochlorococcus* in marine ecosystems; ecosystem models and ecophysiological interpretations were guided by the assumption that most, if not all, *Prochlorococcus* were incapable of nitrate assimilation (García-Fernández *et al.*, 2004; Fuller *et al.*, 2005; Follows *et al.*, 2007). Why have nitrate-utilizing *Prochlorococcus* appeared so infrequently in culture collections in the past? Is it because we were selecting against them in isolations using media containing ammonium but not nitrate (Moore *et al.*, 2007)? We think not because SB and MIT0604—both *narB*-containing strains—grow at equal or better rates on ammonium compared with other HL adapted *Prochlorococcus* strains (Figure 1 and Supplementary Figure S1). An alternative explanation is that most of the early cultures of *Prochlorococcus* were isolated from environments that are relatively nitrogen replete—that is, thought to be more limited by phosphorus or iron availability (for example, the Sargasso Sea, Mediterranean Sea and the Equatorial Pacific) (Vaulot *et al.*, 1996; Mann and Chisholm, 2000; Wu *et al.*, 2000; Marty *et al.*, 2002; Kettler *et al.*, 2007; Rusch *et al.*, 2010). We now know that *Prochlorococcus* cells capable of nitrate assimilation are more likely to be found in ocean regions with lower average nitrate concentrations, such as the Caribbean Sea and Indian Ocean (Martiny *et al.*, 2009b). Indeed, PAC1 and SB (both *narB*-containing strains that were isolated on medium containing ammonium but lacking nitrate) were isolated from N-poor regions (Shimada *et al.*, 1995; Penno *et al.*, 2000; Wu *et al.*, 2000; Iwata *et al.*, 2005). Thus, we believe that the probability of obtaining a *narB*-containing strain using medium containing ammonium is in large part a function of the particular water sample used to start enrichment cultures.

Nitrate assimilation is found in diverse lineages of *Prochlorococcus*

What can the features of the nitrate assimilation genes in *Prochlorococcus* tell us about how they have been gained or lost during the evolution of this group? The genomes of PAC1, SB and MIT0604, along with contigs containing nitrate assimilation genes from the P0902-H212 and P0903-H212 enrichment cultures, were informative in this regard. These *Prochlorococcus* belong to both the LL adapted LLI clade (PAC1, P0902-H212 and P0903-H212) and the HL adapted HLII clade (SB and MIT0604) (Figure 2 and Supplementary Figures S2 and S3), demonstrating that nitrate utilization is found in multiple and diverse lineages of *Prochlorococcus* and suggesting a complex evolutionary history. The presence of nitrite and nitrate metabolism in *Prochlorococcus* follows that of *Synechococcus* in that some strains are able to reduce nitrite and some are able to reduce both nitrite and nitrate. Because these traits are not monophyletic, a model of gene gain and loss events provides evidence for three gains and two losses for the *narB* nitrate reductase gene and two gains and three losses for the *nirA* nitrite reductase gene (Figure 2). With the limited number of genomes available, it appears that there is evidence for multiple gains and losses of nitrogen assimilation traits through the evolution of *Prochlorococcus* and *Synechococcus*, with *narB* found in at least three distinct *Prochlorococcus* lineages.

The genomic context of the nitrate assimilation gene cluster suggests a complex evolutionary history

To look for features that might help us interpret the gains and losses of nitrate and nitrite assimilation genes in *Prochlorococcus*, we examined the local genomic context of these genes. Although the full complement of nitrate assimilation genes was predicted to be localized in a single region of the highly syntenic HLII clade genomes from metagenomic assemblies (Martiny *et al.*, 2009b), it was unclear whether this context would be found in any individual cell. Furthermore, given that these genes were found in a different region in *Prochlorococcus* compared with marine *Synechococcus*, we were curious as to whether we might find evidence for rearrangements or lateral gene transfer.

The nitrate assimilation genes in PAC1 and the P0902-H212 and P0903-H212 contigs are syntenic and also found in the same genomic region as the nitrite assimilation genes in NATL1A and the nitrate assimilation genes in *Synechococcus* WH8102 (Figure 3). This region is bounded by a pyrimidine biosynthesis gene (*pyrG*) and a polyphosphate kinase gene (*ppk*) between which many nitrogen assimilation genes are located in marine *Synechococcus*. Although gene gains and losses have been observed in this region (Scanlan *et al.*, 2009), our data indicate that the genomic location of the nitrate

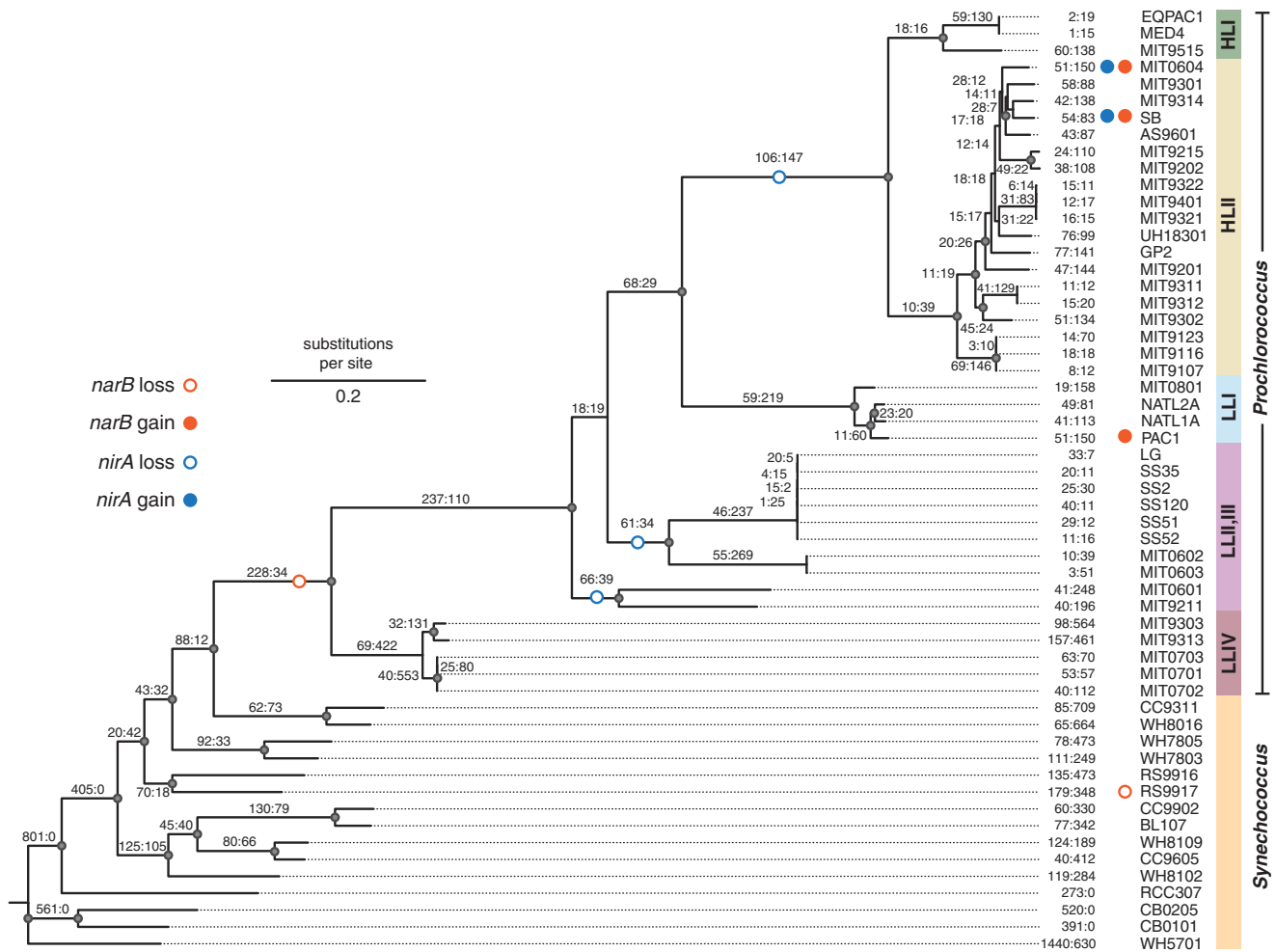


Figure 2 Maximum likelihood phylogeny of *Prochlorococcus* and *Synechococcus* based on the similarity of 100 randomly concatenated single-copy core genes. Nodes are marked by closed circles to indicate that the associated taxa clustered together in at least 75% of 100 replicate trees. Genes lost and gained in the evolution of *Prochlorococcus* and *Synechococcus* are indicated at each node by values representing losses followed by gains. Predicted losses (open circles) or gains (closed circles) of *nirA* (blue) or *narB* (orange) are labeled on their respective branches.

and nitrite assimilation genes is reasonably well fixed in LLI *Prochlorococcus* and closely related *Synechococcus*. Although our model of gene gain and loss events suggests the loss of nitrate assimilation genes early in the evolution of *Prochlorococcus* (Figure 2), the local genomic features of these genes are consistent with the interpretation that some lineages may have retained these genes following the divergence of *Prochlorococcus* from *Synechococcus*.

Analysis of metagenomic data from GOS (Martiny *et al.*, 2009b) suggested that the nitrate utilization genes in HLII *Prochlorococcus* should be located in a different genomic region compared with LLI genomes, indicating an alternative evolutionary origin. Based on a scaffold of mate-paired metagenomic reads, it was inferred that this cluster should be located ~500 kb downstream of the *pyrG-ppk* region containing the nitrate assimilation genes in WH8102 and the nitrite assimilation genes in NATL1A (Martiny *et al.*, 2009b). We found a high

degree of similarity between the nitrate assimilation gene cluster in SB and the scaffold derived from GOS metagenome sequences obtained from multiple individual cells from multiple sampling stations. This similarity manifested itself not only in the gene order and chromosomal location, but also the phylogeny of the nitrate assimilation genes (Figures 3–5), placing the nitrate assimilation gene cluster in a genomic region that is syntenic with other HLII genomes and adjacent to a known genomic island (ISL3) in this clade (Figure 4). Furthermore, a partial genome from a *Prochlorococcus* single cell belonging to the HLII clade (B241-528J8; Genbank JFLE01000089.1) (Kashtan *et al.*, 2014) also possesses a nitrate assimilation gene cluster in the same location and in the same order. The striking similarity between the nitrate assimilation gene clusters of these individual *Prochlorococcus* and the GOS consensus indicates that the order and location of nitrate assimilation genes are stable within HLII genomes.

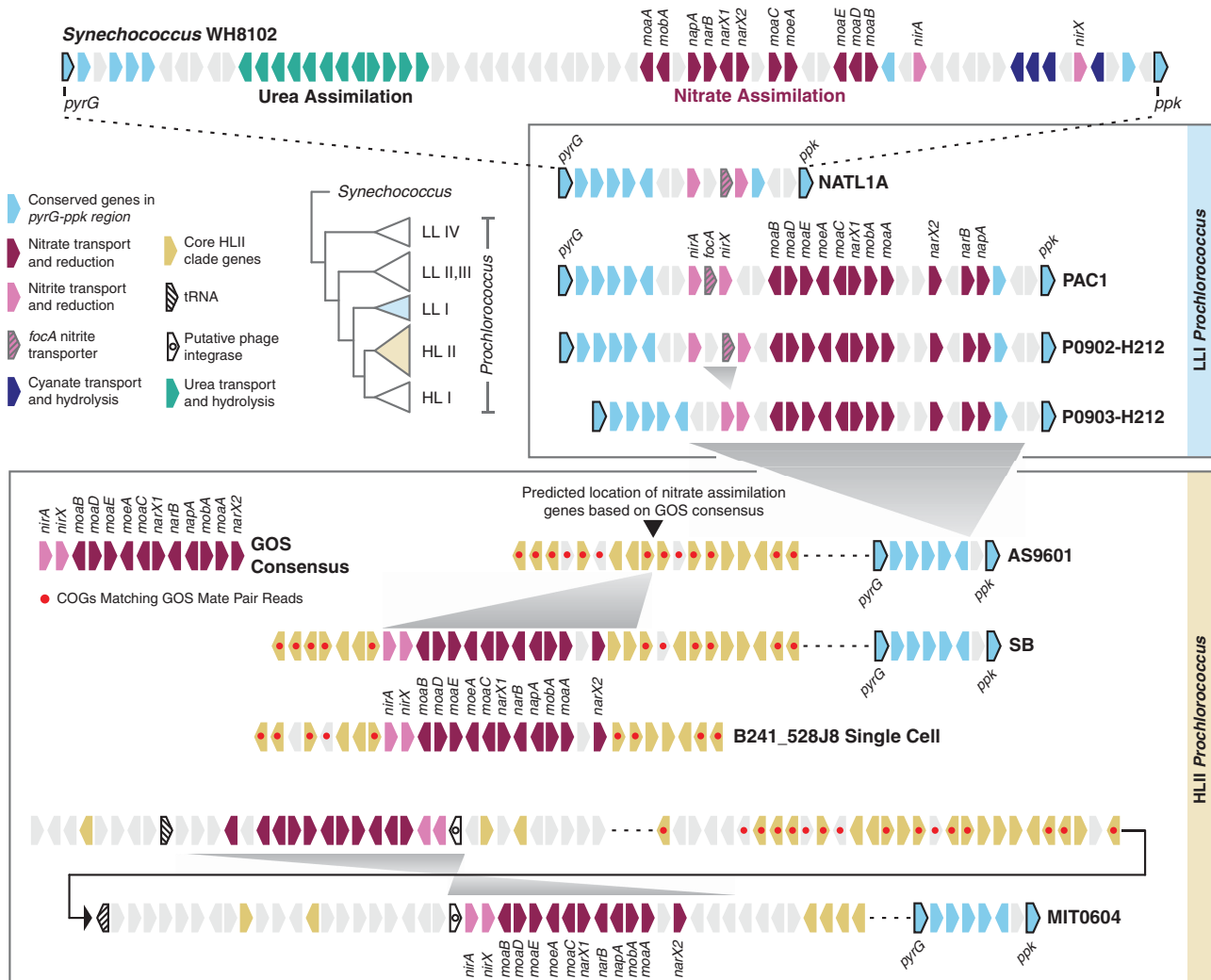


Figure 3 Architecture of the nitrite and nitrate assimilation genes in LL adapted (LLI clade) and HL adapted (HLII clade) *Prochlorococcus* relative to *Synechococcus* WH8102. Similar to *Synechococcus*, the nitrite and nitrate assimilation genes in the LLI clade of *Prochlorococcus* are found within the region between the *pyrG* (pyrimidine biosynthesis) and *ppk* (polyphosphate kinase) genes. Most LLI clade *Prochlorococcus*, with the exception of the P0903-H212 contig, possess a *focA* nitrite transporter in this region (possibly acquired from proteobacteria (Rocap et al., 2003)). Metagenome data (Martiny et al., 2009b), a partial genome from a single cell (B241-528J8) (Kashtan et al., 2014) and a culture genome (*Prochlorococcus* SB) indicate that the nitrate assimilation genes within HLII clade *Prochlorococcus* are commonly found in a syntenic region adjacent to genomic island ISL3 (see Figure 4). *Prochlorococcus* MIT0604 is an exception in that it possesses duplicate nitrate assimilation gene clusters located within genomic islands ISL3 and ISL4 (see Figure 4), with phage integrase genes immediately adjacent to each copy of the *nirA* (nitrite reductase) gene.

The nitrate assimilation genes in strain MIT0604 had a different local genome structure compared with strain SB and the partial single-cell genome, B241-528J8. MIT0604 has duplicate clusters of these genes that are inversely oriented and located upstream and downstream of the GOS-predicted location (Figure 3 and 4). A Southern blot confirmed that MIT0604 does indeed contain two copies of *narB* whereas SB contains only one (Supplementary Figure S4), and they are located within genomic islands ISL3 and ISL4 of HLII clade *Prochlorococcus* (Figure 4). Genomic islands are common features of *Prochlorococcus* genomes, particularly within the HL adapted clades (Coleman et al., 2006; Kettler et al., 2007). They harbor much of the variability in gene content between members of the same clade

and are hot spots for lateral gene transfer. Phage integrase genes are located proximal to both nitrate assimilation gene clusters in MIT0604, and a transfer RNA gene is adjacent to one of these clusters (Figure 3). The transfer RNA genes are known to serve as sites for insertion of phage DNA in bacteria (Williams, 2002), and thus the location of these phage integrase and transfer RNA genes suggests transduction as a possible mechanism by which MIT0604 has acquired the nitrate assimilation gene cluster. Notably, duplication of such a large region of the chromosome has not been observed previously in *Prochlorococcus*, and, thus far, MIT0604 is the only *Prochlorococcus* or *Synechococcus* strain possessing two complete copies of the genes required for nitrate assimilation.

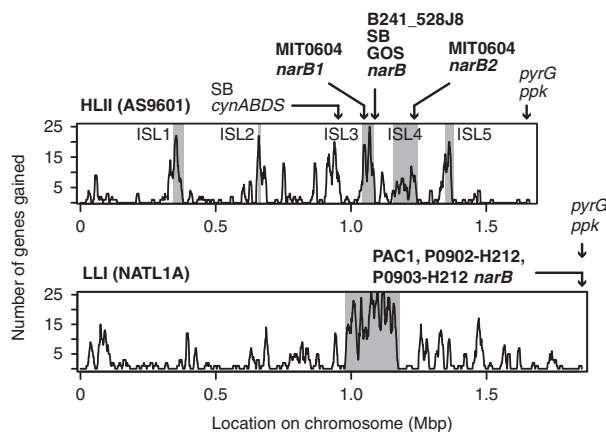


Figure 4 Locations of nitrate and cyanate assimilation genes in strains of *Prochlorococcus* capable of nitrate assimilation relative to the known genomic islands (shaded regions) observed in the HLII and LLI clades of *Prochlorococcus*; plots modified from Kettler *et al.* (2007). *Prochlorococcus* genomes are highly syntenic and genomic islands have been identified in HL adapted genomes (for example, AS9601) by conserved breaks in gene synteny among strains (Coleman *et al.*, 2006; Kettler *et al.*, 2007). Genomic islands have also been identified (for example, the large region within LLI clade genomes such as NATL1A) by predicted gene gain events along the chromosome (Kettler *et al.*, 2007).

The phylogenies of nitrate assimilation genes are similar to the phylogeny of genomes

Given the evidence for both a stable arrangement of the nitrate assimilation genes in some *Prochlorococcus* and possible gene transfer leading to acquisition of the nitrate assimilation trait in MIT0604, we were curious to know whether the phylogenies of these genes were congruent with whole genome phylogenies (Figure 2 and Supplementary Figure S2), as well as the phylogeny of GyrB (Supplementary Figure S3) that has been identified as a useful phylogenetic marker for *Prochlorococcus* (Mühling, 2012). Thus, we reconstructed the amino acid phylogenies of the NirA and NarB reductases, the FocA nitrite transporter and the NapA nitrite/nitrate transporter (Figure 5). The NirA phylogeny is largely consistent with our observations based on the GOS metagenome data (Martiny *et al.*, 2009b), such that the NirA proteins from genomes in the LLIV clade are more closely associated with marine *Synechococcus* than with other *Prochlorococcus* sequences. In all phylogenetic trees, the PAC1, P0902-H212 and P0903-H212 sequences are in a separate clade distinct from that of the SB and MIT0604 sequences, reinforcing the HL versus LL differentiation (Figure 5). The NirA and NarB sequences from SB are consistently more closely affiliated with the GOS consensus sequence (Martiny *et al.*, 2009b) than with the MIT0604 sequences. NapA sequences from SB and MIT0604 are also both closely related to the GOS NapA consensus sequence (Figure 5). Similar to the GyrB phylogeny (Supplementary Figure S3), the P0903-H212 sequences fall outside the clade containing the other LLI sequences. With the exception of the LLIV

NirA sequences, the phylogenies of these nitrite and nitrate assimilation proteins (Figure 5) are congruent with whole genome and GyrB phylogenies (Figure 2 and Supplementary Figures S2 and S3) at a resolution defining the major *Prochlorococcus* clades.

Nitrate assimilating Prochlorococcus possess a diverse set of nitrogen acquisition pathways

Gene content in *Prochlorococcus* has been shown, for several traits, to reflect the selective pressures in the specific environments from which they (or their genes) were captured (Martiny *et al.*, 2006; Rusch *et al.*, 2007; Coleman and Chisholm, 2010; Feingersch *et al.*, 2012; Malmstrom *et al.*, 2013). Thus, we wondered whether other nitrogen assimilation traits might co-occur with nitrate assimilation in *Prochlorococcus*, and examined the potential for PAC1, SB and MIT0604 to access alternative sources of nitrogen based on their gene content (Supplementary Table S1 and Supplementary Figure S5).

Like other members of the LLI clade, PAC1 possesses genes for the assimilation of ammonium and urea, but lacks cyanate transporter genes. In addition to the *napA* nitrite/nitrate transporter, the *focA* nitrite transporter is found in both PAC1 and the contig from P0902-H212. However, the *focA* gene is absent from HL adapted strains SB and MIT0604, and most surface water metagenomic samples (Martiny *et al.*, 2009b). Some *Synechococcus* strains (for example, WH8102) (Supplementary Figure S5) also lack *focA*; thus, this gene is clearly subject to gain and loss. Although *focA* is also similar to formate transporters, evidence implicates its role in nitrite uptake in *Prochlorococcus*; for example, the gene is located near other nitrite assimilation genes (Figure 3), it is upregulated under nitrogen stress (Tolonen *et al.*, 2006) and it is absent from *Prochlorococcus* that cannot grow on nitrite (Moore *et al.*, 2002; Coleman and Chisholm, 2007; Kettler *et al.*, 2007) (Supplementary Figure S5). As PAC1 possesses both a nitrite transporter (*focA*) and the dual-function nitrate/nitrite transporter (*napA*), it is possible that *focA* provides some advantage to LL adapted cells that are often maximally abundant near the nitrite maxima in the oceans (Scanlan and West, 2002; Lomas and Lipschultz, 2006). LL adapted cells that possess the dual-function nitrite/nitrate transporter may benefit from having an additional transporter for nitrite. Given that HL adapted *Prochlorococcus* strains capable of nitrate utilization lack the *focA* gene, these cells may be less reliant on nitrite as a nitrogen source.

SB and MIT0604 possess urea assimilation genes and can utilize urea as a sole nitrogen source (Supplementary Figure S6). Furthermore, SB possesses cyanate transporter genes that are rare in both *Prochlorococcus* and *Synechococcus* strains (Kamennaya *et al.*, 2008), and it can indeed grow

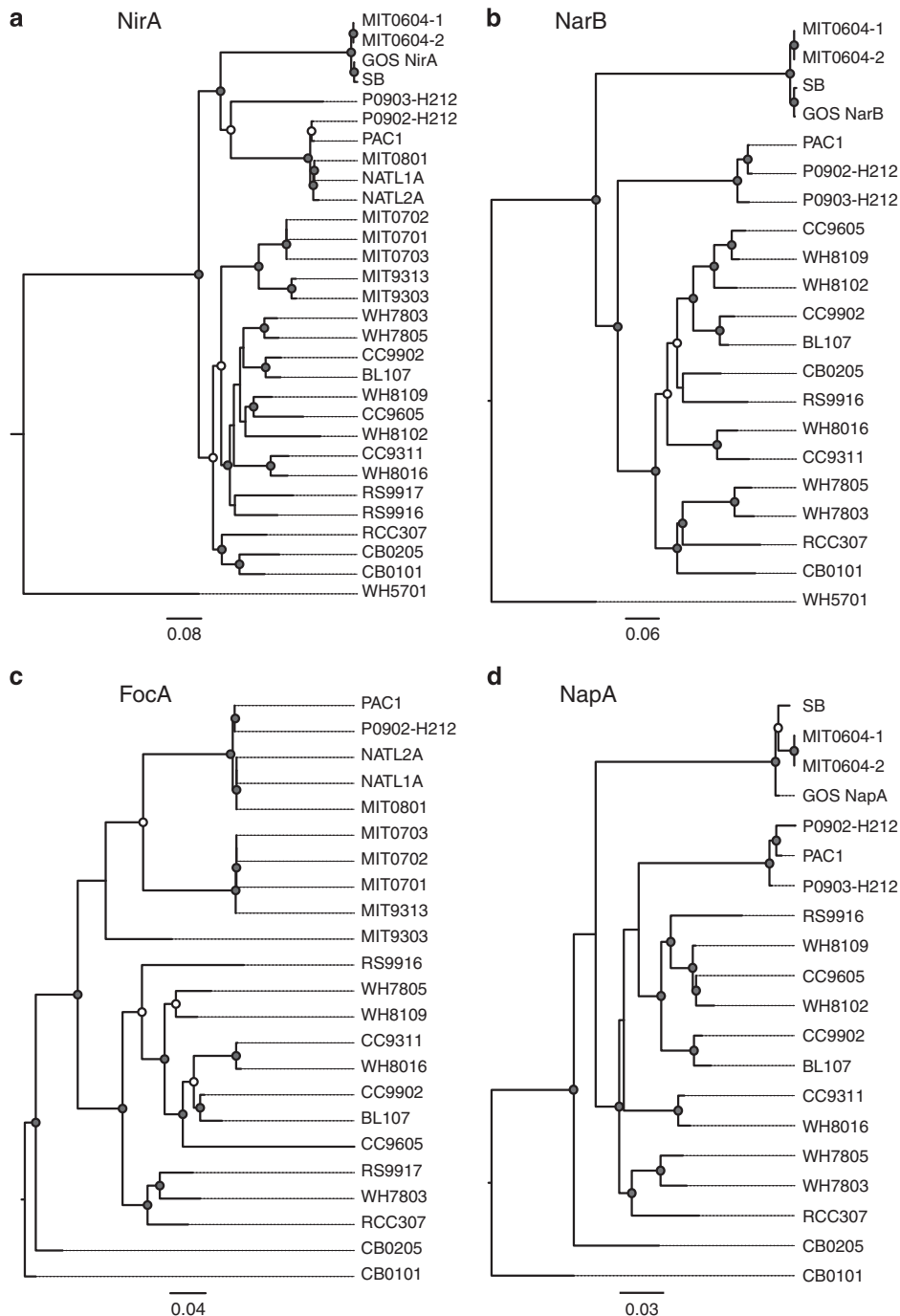


Figure 5 Neighbor-joining phylogeny of four proteins involved in the transport and reduction of nitrate and nitrite in marine cyanobacteria: (a) NirA; nitrite reductase, (b) NarB; nitrate reductase, (c) FocA; nitrite transporter and (d) NapA; nitrite/nitrate transporter. The percentage of 100 replicate trees in which the associated taxa clustered together is indicated at nodes by closed circles (>75%) or open circles (>50%). Scale bars represent substitutions per site.

utilizing cyanate (Supplementary Figure S1) as the sole source of nitrogen. Although very little is known about cyanate concentrations in marine systems, *cynA* genes (encoding the periplasmic component of the cyanate ABC-type transporter system) were relatively abundant in the seasonally stratified and nitrogen depleted waters of the northern Red Sea (Kamennaya *et al.*, 2008). The *cynA* gene of SB clusters with clones obtained from the

Red Sea (Supplementary Figure S7), supporting their origin in HLII clade genomes as hypothesized by Kamennaya *et al.* (2008).

SB contains the most extensive suite of nitrogen acquisition pathways of any cultured *Prochlorococcus* strain examined to date. Why might this be? A useful analogy can be drawn from our understanding of selection pressures that have shaped *Prochlorococcus* genomes with respect to

adaptations involved in phosphorus assimilation. Individual cells and populations from phosphorus-limited environments possess accessory phosphorus acquisition genes, such as alkaline phosphatase (*phoA*) and phosphonate utilization (*phnYZ*) genes, at a higher frequency than *Prochlorococcus* from phosphorus-replete environments (Martiny *et al.*, 2006, 2009a; Coleman and Chisholm, 2010; Feingersch *et al.*, 2012). Thus, we hypothesize that the nitrogen assimilation traits present in *Prochlorococcus* SB were likely shaped by frequent nitrogen limitation in its original habitat (Iwata *et al.*, 2005); that is, cells capable of accessing a wide pool of nitrogen compounds may be at a selective advantage in nitrogen-limited environments.

Conclusions

Given the large standing stock of *Prochlorococcus* in the subtropical oceans and the extent to which nitrogen limits primary production in these regions (Tyrrell, 1999; Moore *et al.*, 2013), the absence of nitrate assimilation capabilities in cultured strains of *Prochlorococcus* has long puzzled biological oceanographers. This motivated field studies (Casey *et al.*, 2007; Martiny *et al.*, 2009b) and the use of models to help us understand the selection pressures driving the loss of nitrate assimilation genes in *Prochlorococcus* relative to *Synechococcus* (Bragg *et al.*, 2010). In this study we show unequivocally that some strains of *Prochlorococcus* are indeed capable of growth using nitrate as the sole nitrogen source. Future studies of these strains will help elucidate the physiological tradeoffs of carrying these genes and help refine the nitrogen inventory in biogeochemical models of the global ocean (Follows *et al.*, 2007). Correlations between environmental nitrate concentrations and ribotype phylogeny (Martiny *et al.*, 2009c) and the striking similarity between *Prochlorococcus* SB and the GOS consensus sequence both suggest that the trait for nitrate assimilation could be tied to distinct ribotype lineages. Still, evolution has many ways of introducing genomic complexity: the MIT0604 genome suggests that these genes are also subject to horizontal gene transfer, allowing further diversification of this trait in other lineages. This is reminiscent of the phylogenetic characteristics of phosphorus acquisition traits that are nearly independent of ribotype phylogeny (Martiny *et al.*, 2009c)—with extensive diversity in the ‘leaves of the tree’. As we learn more about these layers of diversity, it will inform parameterizations of the relationship between light, temperature and nutrient acquisition traits for ocean simulation modeling.

Conflict of Interest

The authors declare no conflict of interest.

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