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Culture Isolation and Culture-Independent Clone Libraries Reveal New Marine *Synechococcus* Ecotypes with Distinctive Light and N Physiologies▽

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Marine microbial communities often contain multiple closely related phylogenetic clades, but in many cases, it is still unclear what physiological traits differentiate these putative ecotypes. The numerically abundant marine cyanobacterium *Synechococcus* can be divided into at least 14 clades. In order to better understand ecotype differentiation in this genus, we assessed the diversity of a *Synechococcus* community from a well-mixed water column in the Sargasso Sea during March 2002, a time of year when this genus typically reaches its annual peak in abundance. Diversity was estimated from water sampled at three depths (approximately 5, 70, and 170 m) using both culture isolation and construction of cyanobacterial 16S-23S rRNA internal transcribed sequence clone libraries. Clonal isolates were obtained by enrichment with ammonium, nitrite, or nitrate as the sole N source, followed by pour plating. Each method sampled the in situ diversity differently. The combined methods revealed a total of seven *Synechococcus* phylotypes including two new putative ecotypes, labeled XV and XVI. Although most other isolates grow on nitrate, clade XV exhibited a reduced efficiency in nitrate utilization, and both clade XV and XVI are capable of chromatic adaptation, demonstrating that this trait is more widely distributed among *Synechococcus* strains than previously known. Thus, as in its sister genus *Prochlorococcus*, light and nitrogen utilization are important factors in ecotype differentiation in the marine *Synechococcus* lineage.

As molecular methods have been applied to explore the structures of vastly diverse microbial communities (42, 50, 66), one pattern that has emerged is that of microdiversity—the coexistence of closely related but distinct phylogenetic clades (1, 28, 66). It has been hypothesized that these clades represent physiologically and ecologically distinct populations or ecotypes (9, 28, 46). However, physiological differences and the ecological distribution of populations have not been explored for most communities at the ecotype scale, in part due to difficulties in cultivating many microbes (50). Thus, the mechanisms that allow for the coexistence of closely related phylogenetic clades are largely unknown. The coexistence of multiple taxa, all fulfilling the same functional ecological role, has also been of longstanding interest to marine ecologists (25). In comparison to physically stratified environments such as sediments, or microbial mats, which often have clear gradients in abiotic parameters (17, 19), the dynamic marine water column presents a greater challenge to model the mechanisms driving ecotype differentiation.

The cyanobacterium *Synechococcus* is an attractive model system for exploring ecotype differentiation in marine habitats because it is easily enumerated, and numerous clonal isolates representing distinct closely related phylogenetic clades have been cultivated (18, 21, 51, 64, 68). Furthermore, these clades have been repeatedly observed to coexist in a single water column (7, 8, 21, 22, 30, 35, 44). *Synechococcus* inhabits the world's coastal and oligotrophic oceans at high concentrations (typically 1×10^3 to 1×10^4 cells ml^{-1}), and in the oligotrophic Atlantic, it comprises a significant portion of phytoplankton carbon (13), accounting for up to a quarter of primary production (29). Most marine *Synechococcus* strains belong to marine cluster 5.1 (previously called marine cluster A), a genus-level taxon within the provisional form-genus *Synechococcus* (24a). Marine cluster 5.1 (hereafter referred to as the genus marine *Synechococcus*) contains at least 10 distinct phylogenetic clades identified from cultured isolates using 16S rRNA genes (21). Some of these clades possess distinguishing physiological characters that render them ecologically distinct. For example, strains belonging to clade III possess a unique form of motility that is absent in all other marine *Synechococcus* strains (64, 69). Members of clades I and III are chromatically adaptive; they can alter the relative ratio of their accessory pigments, phycourobilin (PUB) and phycoerythrobilin (PEB), according to the spectral quality of the light under which they are growing (43). Other clades altogether lack PUB (clade VI) or both PUB and PEB (clade VIII) (68). Differential distribution of particular clades with season as revealed by PCR dot blot hybridization has provided further evidence that certain clades are ecologically distinct (21, 22). However, physiological traits that distinguish many clades remain to be discovered.

Light is known to be an important factor in niche differentiation in cyanobacteria. Closely related taxa of *Cyanobium* isolated from the Baltic Sea possess different accessory pigments that allow them to coexist by partitioning the photosynthetically available spectrum of light (56). Light adaptation is also important in the differentiation of *Prochlorococcus*, the

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sister clade to marine *Synechococcus* with which it coexists in oligotrophic waters. *Prochlorococcus* ecotypes fall into two major groups according to adaptation to intensity and spectral quality of light, and ecotypes belonging to these two groups partition the upper and deep euphotic zones accordingly (2, 37, 70, 73). *Synechococcus* ecotypes exhibit differences in their accessory pigments (see above) that affect adaptation to spectral light quality, but adaptation to high or low light has not been thoroughly investigated in *Synechococcus* strains as it has for *Prochlorococcus* strains. Strain CC9605 (clade II) serotype cells quantified by immunofluorescence are more abundant in surface mixed waters than at depth off the coast of California, suggesting that this ecotype may be better adapted to higher-light conditions (63). In contrast, in the Red Sea, where clade II is the dominant genotype, depth partitioning has not been seen (22).

N is also an important factor in niche differentiation in marine microbes because both its concentration and its form vary in marine ecosystems (11, 33). For example, a strong correlation between nitrate concentration and patterns in the diversity of heterotrophic bacteria along a transect in the arctic North Atlantic suggests that nitrate availability shapes microbial community structure in these waters (3). N probably plays a similar role in shaping open-ocean communities, especially since it is thought to often be limiting (65). In the Sargasso Sea, N availability is driven by seasonal patterns of mixing and stratification and dynamic changes in biota (13, 23, 33). In this region, the annual peak of marine *Synechococcus* occurs in late spring, a time when standing stocks of nitrate are also at a maximum as a result of recent deep winter mixing (13, 33). Marine *Synechococcus* strains cannot fix N_2 but are capable of using a wide variety of organic (urea and amino acids) and inorganic (ammonium, nitrite, and nitrate) N sources (38, 45, 68). In cyanobacteria, nitrate is assimilated through a reductive pathway mediated by nitrate (*narB*) and nitrite reductase (*nirA*), so strains capable of using nitrate can also use nitrite (20). Variability in inorganic N utilization is important in niche differentiation in *Prochlorococcus* ecotypes (38); however, the role of N in ecotype differentiation in the genus marine *Synechococcus* has not been thoroughly explored. The majority of *Synechococcus* strains in culture are capable of using nitrate as a sole N source, but most of these strains were originally isolated using nitrate (21, 38, 68). Several strains recently isolated using ammonium are incapable of growing on nitrate: a single strain, MITS9220, belonging to the newly described clade CRD2, and members of clade VIII (21, 38, 54). Therefore, it is possible that the total diversity of this genus is underrepresented in cultured isolates because of a selective bias for strains that can grow on nitrate.

Molecular evidence, including the recent discoveries of at least four new phylogenetic clades, also suggests that the total diversity within the genus marine *Synechococcus* remains to be fully explored (21, 30, 47, 54). In addition, quantification of the relative abundance of particular clades of *Synechococcus* in the Red Sea reveals that in some cases, the summation of abundances for known clades does not fully account for total community abundance (21, 22). Sequencing of the 16S-23S rRNA internally transcribed spacer (ITS) from the Costa Rica Dome reveals one clade for which there are no known cultured isolates (54). Clone libraries of RNA polymerase sequences (*rpoC1*) and of the nitrogen regulation gene (*ntcA*) also suggest that more *Synechococcus* clades exist than are currently in culture (18, 30, 47, 62). Further characterization of the extant genetic diversity and the physiological diversity associated with clades is key to understanding their ecology and the mechanisms of differentiation with this genus.

In this study, we sought to better understand the role that multiple ecotypes play in the ecological success of the genus marine *Synechococcus* during their annual peak in abundance by simultaneously using culture isolation and environmental sequence clone libraries to assess diversity. Isolations were done with ammonium, nitrite, and nitrate in order to further explore the diversity of N physiology. We characterized the N and light physiologies of the isolates obtained to better understand the importance of these parameters in ecotype differentiation in this genus. We also compared the effectiveness of both culture-dependent and -independent approaches in estimating the total diversity of the community.

MATERIALS AND METHODS

Sample collection. Water samples were taken from the western Sargasso Sea in March 2002. Standard hydrographic data and water samples were collected using an SBE19 Seabird Electronics (Bellevue, WA) conductivity-temperature-depth (CTD) package mounted with 12-liter Niskin bottles. Water was taken at 5, 70, and 170 m for culture isolation using acid-clean Go-Flo bottles on 23 March at 9:20 a.m. (local time) (34°11′N, 70°12.5′W). While following a drogue set at 5 m, water for clone library construction was taken from the CTD rosette at 5, 75, and 175 m on 25 March (5:21 a.m., local time) (33°59.8′N, 70°13.8′W).

Flow cytometry. Duplicate water samples from the CTD cast were preserved with glutaraldehyde (0.125%) and stored in liquid nitrogen for flow cytometric analysis. Populations of picophytoplankton were identified by their distinct flow cytometric signatures (41) and enumerated using a FACScan flow cytometer (Becton Dickson Biosciences, Rockville, MD) and 2-μm beads (Polysciences, Warrington, PA) as an internal standard. Abundances were determined on single samples, since the accuracy of cell abundance determined by flow cytometry is typically within a few percent (4).

ITS clone libraries. Water samples for cyanobacterial ITS clone libraries were first prefiltered through 20-μm mesh, and cells from 100 ml of water were then collected onto 25-mm 0.2-μm polycarbonate Poretics filters (GE Osmonics, Minnetonka, MN) with less than 15 in. Hg of vacuum. Filters were chased with 3 ml preservation solution (0.5 M NaCl, 10 mM Tris [pH 8.0], 100 mM EDTA [pH 8.0]) (70), placed into 2-ml bead-beating tubes (BioSpec Products Inc., Bartlesville, OK), flash-frozen in liquid N_2, and stored at −80°C. Genomic DNA was purified using the QIAGEN (Valencia, CA) DNeasy tissue kit according to the protocol provided by the manufacturer for gram-negative bacterial cells. Filters were first vortexed vigorously with lysis buffer using a bead beater (BioSpec Products Inc.) for 30 s at 5,000 rpm, and the buffer with cells was then processed according to the manufacturer's protocol.

Cyanobacterial 16S-23S rRNA ITS regions were PCR amplified in 25-μl reaction mixtures containing 0.25 mM each deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 0.5 μM each primers 16S-1247f and 23S-241r (Table 1), 1 U *Taq* polymerase (Promega, Madison, WI), 1× buffer provided with the polymerase, and 1 to 2 μl each of genomic DNA template. Cycling conditions were as follows: 5 min at 95°C; *n* cycles (see below) of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C; and 10 min at 72°C. In order to minimize PCR biases that may occur in the stationary phase of PCR product amplification, real-time PCR was utilized to select the number of cycles for which amplification was still in the logarithmic phase. Reactions for the 5-, 75-, and 175-m samples were run for 22, 22, and 24 cycles, respectively. To minimize cloning of heteroduplex PCR artifacts, initial reaction mixtures were "reconditioned" (60) by transferring 2.5 μl each of the initial reaction mixture into 22.5 μl of fresh PCR mixture and run for an additional three cycles followed by a 10-min extension step at 72°C.

PCR products were purified with a QIAGEN PCR purification kit and cloned using a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA). Approximately 30 colonies from each depth were picked for sequencing using plasmid DNA prepared with a QIAGEN Plasmid Miniprep kit or from DNA amplified using a Templiphi kit (Amersham Biosciences Corp., Piscataway, NJ). Amplified or purified plasmid DNA was used in 10-μl sequencing reaction mixtures with a

TABLE 1. Primers used for PCR and sequencing of the 16S-23S ITS and *narB*

Primer	Sequence (5′–3′)	Reference or source
PCR primers		
16S-1247f	CGTACTACAATGCTACGG	51
23S-241r	TTCGCTCGCCRCTACT	51
narBF1	GAAGGCAAYCCGATGTGGA	This work
narBdegR1	GCCCCTACTGCGGTGTNGGCT GYGG	This work
Sequencing primers		
ITS-AlaF	TWTAGCTCAGTTGGTAGAG	51
ITS-AlaR	CTCTACCAACTGAGCTAWA	51
narBseq1F	GACATCCACTTGCCGATTG	This work
narBseq1R	CAATCGGCAAGTGGATGTC	This work
narBseq2F	CCCARTGGAGYGAGAAGGC	This work
narBseq2R	GCCTTCTCRCTCCAYTGGG	This work
narBseq3F	AACAATTCAGCTTCGATTCAG	This work
narBseq3R	CTGAATCGAAGCTGAATTGTT	This work
narBseq4F	AGCAGRTSGAAGCRATGGA	This work
narBseq4R	TCCATYGCTTCSAYCTGCT	This work
narBseqAF	ATTGCWCCRGGCAGCG	This work
narBseqAR	CGCTGCCYGGWGCAAT	This work

DYEnamic ET dye terminator kit (Amersham Biosciences) according to the instructions provided by the manufacturer and run on a MegaBACE 1000 automated sequencer (Amersham Biosciences). The program Sequencher (Gene Codes Inc., Ann Arbor, MI) was used to align sequence chromatographs and manually check base calls. Cloned ITS sequences were screened by sequencing one read using primer 16S-1247f (Table 1) or M13 primers targeting sites in the cloning vector. The full ITS was sequenced using M13, ITS PCR, and internal sequencing primers (Table 1) for 15 clones representing putative novel *Synechococcus* clades (≤95% sequence identity to previous ITS sequences) or for cyanobacterial clades that are poorly represented in sequence libraries.

Isolation and culture conditions. After prefiltering through 20-μm mesh, seawater was gravity filtered through either a 0.8- or 2-μm polycarbonate filter (Poretics) into an acid-washed glass flask. Filtrate was amended with the following nutrients: 10 μM NaH_2PO_4, trace metal and EDTA mixture as described previously by Moore et al. (38), and 250 μM of either NH_4Cl, $NaNO_2$, or $NaNO_3$. Amended water was split into four replicate tubes and incubated at 20°C under a 12-h light–12-h dark cycle using the following light levels, which approximated the in situ light intensities: 83 to 150, 8 to 16, and 5 to 8 μmol Q m^{-2} s^{-1} at 5, 70, and 170 m, respectively. After being maintained at the above-described conditions for approximately 1 week on board the research vessel, enrichment cultures were switched to a constant light cycle at 20°C and maintained by transferring the cultures into fresh medium approximately every 4 weeks for at least 2 months, after which cultures were pour plated (6) to obtain clonal isolates. For all plating and subsequent culture work, we used an artificial seawater (ASW) medium containing Turks Island salts mix (32), 1 mM HEPES (pH 7.0), 2 mM $NaHCO_3$, trace metal, and EDTA mix as described above (38). For plating, ASW was amended with 10 μM NaH_2PO_4 and 250 μM of the appropriate inorganic nitrogen source (nitrate, nitrite, or ammonium). Plates were made with 0.4% low-melting-point agar (Invitrogen Corp., Carlsbad, CA). Ten microliters of serially diluted culture was added to molten agar maintained at 29°C. For each plating attempt, 10 individual colonies were picked and returned to ASW with 50 μM NaH_2PO_4 and 800 μM $NaNO_3$ for strains isolated on nitrate or 50 μM NaH_2PO_4 and 800 μM NH_4Cl for those isolated on ammonium or nitrite.

Genomic DNA isolation, PCR amplification, and sequencing. Genomic DNA from cultured isolates was purified using a QIAGEN DNeasy tissue kit on 4 to 6 ml of centrifuged cell pellets (6,700 × *g* for 10 to 20 min). The 16S-23S rRNA ITS was PCR amplified from genomic DNA in 25-μl reaction mixtures under the conditions described above, with 35 amplification cycles. Quintuplicate PCRs were pooled and purified with a QIAGEN PCR purification kit before sequencing. The ITS was partially sequenced using primer 16S-1247f (Table 1) to genotype all 89 strains. The full ITS was sequenced for 22 selected strains representing all putative ecotypes.

Primers specific for the amplification of the nitrate reductase gene, *narB*, from marine *Synechococcus* strains were designed using CodeHop (53) based on known *narB* sequences from marine *Synechococcus* strains and other cyanobacteria (Table 1). Twenty-five-microliter PCR mixtures contained 0.25 mM each deoxynucleoside triphosphates, 3.5 mM $MgCl_2$, 0.5 μM each primers narB1F and narBdegR1, 1 U *Taq* polymerase (Promega), and 1× buffer provided with the polymerase. Cycling conditions were as follows: 4 min at 95°C; 40 cycles of 1 min at 95°C, 1 min at 64°C, and 2.5 min at 72°C; and 10 min at 72°C. Quintuplicate reaction mixtures were pooled and purified with a QIAGEN PCR purification kit before sequencing. PCR-amplified *narB* products were sequenced with PCR primers narBF1 and narBdeg1R and internal sequencing primers (Table 1).

Phylogenetic analysis. Additional ITS sequences were obtained online from preliminary releases of genomes at The Institute for Genomic Research (http://www.tigr.org) for strain CC9311 and at the U.S. Department of Energy Joint Genome Institute (www.jgi.doe.gov/) for strains CC9902 and CC9605. Environmental ITS clone sequences from previous studies off the coast of California (MB11A04 and MB11E09) and in the Costa Rica Dome (labeled "CRD") were also included (54, 57). All ITS sequences were aligned using previous alignments of cyanobacterial ITS sequences (51). After the initial alignment, further adjustments were made by taking into account stem-loop structures predicted by MFOLD (74). Loop portions of predicted stem-loop structures and the two tRNAs present in the ITS were excluded. Phylogenetic analysis was performed with PAUP* (59) using 406 positions. The program MODELTEST (48) was used to identify the Tamura-Nei model with a gamma distribution of rate heterogeneity (TrN+G) as the best-fit model for the data. Phylogenetic trees were constructed using either the HKY85 substitution model or the TrN+G model. Best trees were inferred using minimum evolution as the criterion with either of above-described substitution models or using maximum-likelihood methods with the TrN+G model. All three approaches produced trees with similar topologies, except that the best tree found by maximum likelihood (−ln likelihood = 3,983) inferred that clades II and XV do not have a common ancestral node and rather that clade XV is derived from within clade II. However, this tree was not significantly better than the best tree found by constraining clade II strains as monophyletic (−ln likelihood = 3,965) as determined by both the Shimodaira-Hasegawa and Kishino-Hasegawa tests.

For *narB* phylogenetic trees, additional *Synechococcus* sequences for strains CC9605, CC9902, and CC9311 were obtained from the genome sequencing projects listed above. *narB* sequences were provided for the following strains by B. Jenkins: WH 6501, WH 7803, WH 8020, UW92, and UW105 (GenBank accession numbers DQ069071, ADQ069064, DQ069073, DQ075325, and DQ069067) (B. Jenkins and J. Zehr, unpublished results). *narB* nucleotide sequences were aligned according to the translated protein products. Distance-based phylogenetic trees were created with both nucleotide sequences and the predicted amino acid sequences. All nucleic acid positions were included in the analyses, since substitution saturation was not detected using the program DAMBE (72). Nucleotide trees were constructed using PAUP* with the best-fit model, HKY+G, identified by MODELTEST, and minimum evolution as the search criterion. Amino acid trees were inferred using the Fitch-Margoliash criterion on distances computed with the Dayhoff PAM matrix as implemented in PHYLIP (16).

Rarefaction analysis. Estimate*S* was used for rarefaction analysis of clone libraries (version 7 [http://purl.oclc.org/estimates]) (10). ITS clones that were fully sequenced were assigned to distinct clades found in phylogenetic analyses supported by strong bootstrap values and that met the criteria suggested previously by Palys et al. (46): distinct clades are those for which the average distance to the most closely related clade is on average twice that of the average distance between members within the clade. All partially sequenced clones shared >87% identity over 150 bp of the first variable region of the ITS with a fully sequenced clone or isolate and could be unambiguously assigned to a clade.

N utilization experiments. N utilization experiments were conducted to further characterize the physiology of newly isolated strains. Selected strains isolated using ammonium were screened for growth on nitrate. Cultures were grown at 20°C, with approximately 20 μmol Q m^{-2} s^{-1} constant light, in ASW containing 800 μM NH_4Cl and 50 μM NaH_2PO_4 and were then transferred to ASW with 800 μM $NaNO_3$ and 50 μM NaH_2PO_4. Growth was monitored using a fluorometer (Turner Designs, Sunnyvale, CA).

More extensive growth experiments were conducted on strains representing the newly described clade XV. Clade II strain UW86 was used as a positive control. Experiments were repeated three times, twice in light and dark (12 h-12 h) and once in constant light. Cultures were initially acclimated for at least 18 generations in exponential phase in ASW with 100 μM NH_4Cl and 10 μM NaH_2PO_4. Cultures were grown at 20°C with 30 μmol Q m^{-2} s^{-1} light. One milliliter of acclimated culture was transferred into triplicate tubes with 30 ml

ASW containing 10 μM NaH_2PO_4 and either 100 μM NH_4Cl, 100 μM $NaNO_2$, 100 μM $NaNO_3$, or no N. Growth was monitored using a fluorometer, and exponential growth rates were calculated by taking the regression of the natural logarithm of fluorescence versus time. Preserved samples were also taken and counted by flow cytometry as described above.

N recovery experiments were performed to test if the growth deficiency on nitrate was due to a poisoning effect of nitrate at the concentrations used. These experiments were done in constant light. Cells were first acclimated in ASW with 100 μM NH_4Cl and were then transferred to nine tubes of medium containing 100 μM $NaNO_3$. After 4 or 5 days, either no additional N, 100 μM NH_4Cl, or 100 μM $NaNO_2$ was added to triplicate sets of tubes (still containing $NaNO_3$ medium).

Pigment spectra and PUB/PEB ratios. Ratios of the accessory pigments PUB and PEB were determined using spectrofluorometry according to methods described previously by Palenik (43). Cultures were grown at 20°C in ASW with 800 μM NH_4Cl and 50 μM NaH_2PO_4 under ~30 μmol Q m^{-2} s^{-1} constant light. Cultures grown under blue light were placed within a rack enclosed in a Roscolux #69 blue filter (brilliant blue, peak transmittance at 440 nm) (Rosco Laboratories Inc., Stamford, CT) such that the measured light level inside the rack was ~30 μmol Q m^{-2} s^{-1}. Ratios were determined after cultures were acclimated to the growth conditions as determined when growth rates and/or PUB/PEB ratios stabilized, typically at least nine generations. Excitation spectra were obtained from 450 to 560 nm while measuring the emission at 570 nm on a spectrofluorometer (model LS50-B; Perkin-Elmer) using the ratio mode. The excitation and emission slits were both 5 nm, and spectra were measured at a rate of 50 nm per min. The PUB/PEB ratio was calculated using peak fluorescence values near 495 and 595 nm, representing PUB and PEB, respectively.

Absorption spectra were obtained using both in vivo cells and cell lysates using a Spectramax M2 spectrofluorometer (Molecular Devices Corp., Sunnyvale, CA). A sonicator was used to disrupt cells. The lysate was centrifuged for 10 min at 16,000 × *g*, and the absorption spectra on the resulting supernatant were measured.

Nucleotide sequence accession numbers. Full-length ITS sequences of cultured isolates, full-length ITS environmental clone sequences, and *narB* sequences were deposited in GenBank under accession numbers DQ351294 to DQ351316, DQ351317 to DQ351333, and DQ351334 to DQ351338, respectively.

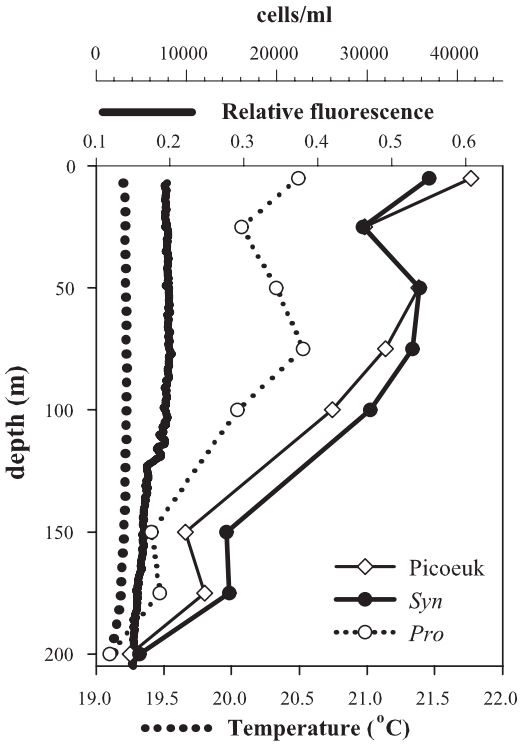

FIG. 1. Hydrographic and cell abundance data for the water column from which samples were taken for the construction of clone libraries. This water column was typical of the Sargasso Sea at the time of sampling. Data for temperature (dotted line), relative chlorophyll fluorescence (solid line), *Synechococcus* cells (filled circles), *Prochlorococcus* cells (open circles), and picoeukaryotic phytoplankton (open diamonds) are shown.

RESULTS

Hydrographic conditions. The water column in the Sargasso Sea at the time of sampling (March 2002) was apparently well mixed down to at least 200 m (Fig. 1). Temperature, density, and chlorophyll fluorescence were nearly uniform over this range, as is typical for this time of year, when a stable mixed layer has not yet been formed (13). During the sampling period, warming of the surface waters was observed during the day, which often resulted in a shallow stratified layer (<35 m) that was absent the following morning. Chlorophyll fluorescence was slightly elevated above 125 m (Fig. 1). This layer was probably caused by repeated daily cycles of growth in warmed surface layers that were mixed down to depth by nightly cooling. Picophytoplankton concentrations were typical for this time of year (13); *Synechococcus* cells were more abundant than *Prochlorococcus* cells at all depths and in equal or higher concentrations than picoeukaryotes, except at 5 m (Fig. 1). All three picophytoplankton groups decreased sharply in abundance below 100 m even though physical parameters indicated that the water column was well mixed.

Diversity of clone library sequences. Clone libraries of cyanobacterial 16S-23S rRNA ITS sequences were created to assess the genetic diversity of the community. A total of 37, 27, and 26 ITS clones were obtained from 5, 75, and 175 m, respectively. Only cyanobacterial sequences were found in the clone libraries. After sequencing clones with a single primer to genotype them, the full ITS was sequenced for 15 clones representing the seven different cyanobacterial clades found, two *Prochlorococcus* clades and five *Synechococcus* clades (Table 2). The concave shapes of rarefaction curves indicate that the cyanobacterial ITS clone libraries at each depth were well sampled (Fig. 2).

Included in the *Synechococcus* clades found were two distinct phylogenetic clades that are supported by high bootstrap values but did not correspond to any closely related sequences in the public database (Fig. 3). Given that 14 other clades of marine *Synechococcus* have been previously described (47), we have labeled these new clades XV and XVI. Sequences in clade XV are closely related (92.8% identity) to previously described clade II sequences, although high bootstrap values support clade XV as being monophyletic. Still, further physiological data were needed to test if this clade is ecologically distinct from clade II. Clade XVI is quite divergent from any other ITS sequence (85.0% identity to clade VI, its most closely related clade) and likely represents an ecotype, given that other clades with less divergence between them are physiologically distinct, such as clades II and III. However, it remained possible that these clades represented the same phylogenetic group as clades identified by sequencing of different loci (16S rRNA, *ntcA*, or *rpoC1*) (18, 21, 30, 47).

In addition to the two potentially novel clades of *Synechococcus*, clades II, IV, and X and *Prochlorococcus* eMIT9312 sequences were also found (Fig. 3). Seven sequences were

TABLE 2. Phylogenetic clades obtained from culture isolation and clone libraries[a]

Phylogenetic clade	Clade obtained at:: 5 m I	5 m C	70 m I	75 m C	170 m I	175 m C	All depths combined I	All depths combined C
Synechococcus								
I	■		■				■	
II		■	■	■		■	■	■
IV		■						
VII	■						■	
X		■		■		■		■
XV	■		■				■	
XVI			■		■	■	■	■
Prochlorococcus[b]								
eMIT9312		■	■	■		■	■	
eNATL2A		■		■			■	■

[a] I, isolates; C, clone libraries.
[b] Ecotype designations are as given in reference 2.

deemed to belong to clade X because they had a high identity (95.5 to 97.5%) to the ITS of clade X strain RCC307 (a derivative of strain Minos01) (21). These sequences were not included in the tree because, like RCC307, they did not align well with the other marine *Synechococcus* ITS sequences.

Genetic diversity of cultured isolates. In an effort to obtain cultured representatives of the two potentially novel clades and to compare culture-dependent and -independent diversity methods, clonal isolates were obtained by enrichment of water with ammonium, nitrite, or nitrate as a sole N source and subsequent pour plating. We obtained 89 clonal isolates from depths of 5, 70, and 170 m. Sequencing of the 16S-23S ITS revealed that strains belonged to the two new phylogenetic clades, XV and XVI, as well as three previously described *Synechococcus* clades (I, II, and VII) (Table 2 and Fig. 3). A single isolate of the *Prochlorococcus* strain eMIT9312 ecotype was also obtained (Table 2). Obtaining cultured isolates of clades XV and XVI allowed us to sequence the 16S rRNA, *ntcA*, and *rpoC1* genes from these strains to determine if clades XV and XVI were the same as clades previously identified using the above-described loci. *rpoC1* sequences from clade XVI strains were >97% identical to a cloned sequence from the Red Sea that has not been explicitly assigned to a particular clade (restriction fragment length polymorphism type S6; GenBank accession number AJ584718) (39). With that exception, sequences for clades XV and XVI from all three loci were distinct from those for previously identified clades (N. Ahlgren and G. Rocap, unpublished data).

Rather than one clade overtaking all other types in enrichment cultures during the period of incubation before pour plating (2 to 3 months), it was a common occurrence that multiple clades coexisted in enrichment cultures. Four out of the nine enrichment culture tubes plated from 5 and 70 m produced isolates from two or more different clades. In contrast, only clade XVI strains were isolated from 170-m enrichments.

Comparison of diversity in culture isolation and clone libraries. Culture isolation and ITS clone libraries sampled the diversity of the cyanobacterial community differently. A total of nine cyanobacterial clades were recovered from the combined use of culture isolation and clone library methods. Five of these clades were seen either only in the isolates or only in the clone libraries, and four clades were found using both methods (Table 2). *Synechococcus* clade II was dominant in both the clonal isolates and the clone library sequences, 49% and 75%, respectively. Although there are potential biases in both clonal isolation and clone libraries, our results, along with data from the Red Sea (21, 22), support the hypothesis that

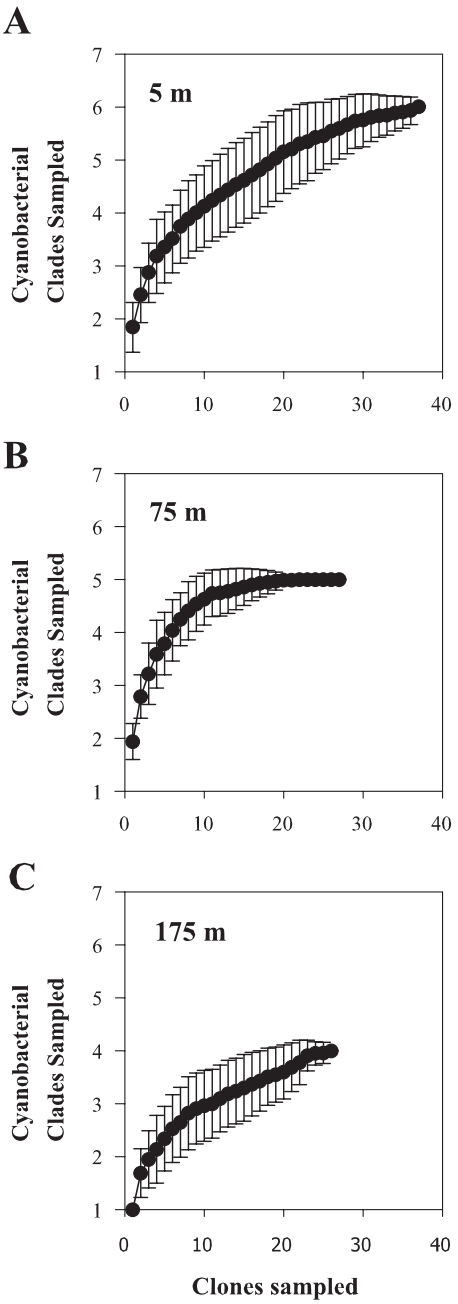

FIG. 2. Rarefaction curves of cyanobacterial phylotype diversity found for clone libraries constructed from depths of 5 m (A), 75 m (B), and 175 m (C). Error bars represent standard deviations of 100 replicate curves.

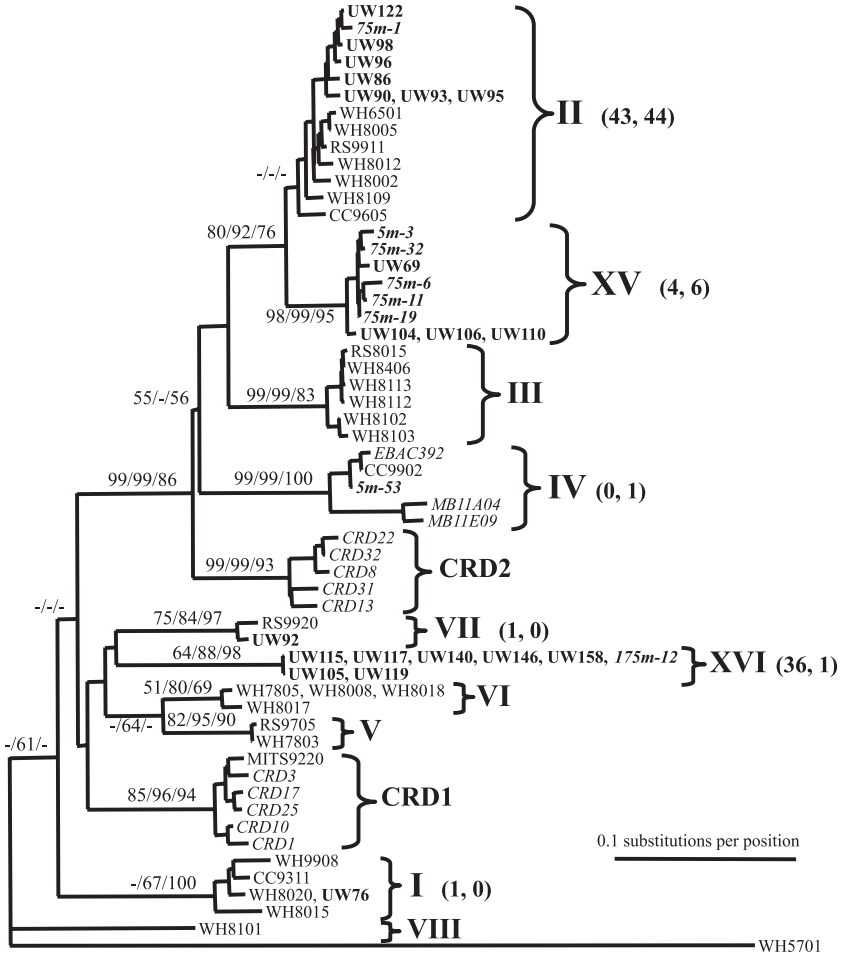

FIG. 3. Phylogenetic tree of the genus marine *Synechococcus* inferred using 406 positions of the 16S-23S ITS region. The tree shown was constructed using the HKY85 model of nucleotide substitution and minimum evolution as the objective, but tree topologies found using other methods were similar (see Materials and Methods). Bootstrap values greater than 50 are given at the nodes and were determined using the HKY85 model with minimum evolution (1,000 replicates), the TrN+G model with minimum evolution (1,000 replicates), and the TrN+G model with maximum likelihood (100 replicates). Sequences from environmental clones are in italics. Sequences obtained in this study are in boldface type. The total numbers of sequences recovered for each clade are listed after the clade number designation (isolates obtained, sequences cloned).

clade II strains dominate in oligotrophic habitats. Similarly, the eMIT9312 ecotype dominated *Prochlorococcus* clones in the libraries at 5 and 75 m and has been found using real-time PCR to be the most abundant *Prochlorococcus* ecotype in the upper waters of the Western North Atlantic (2, 73).

There was no statistically significant difference detected in the pattern of clades recovered in clone libraries by depth ($P = 0.6$; analysis of variance). Considerable overlap was seen in clades recovered at the upper two depths for both clone libraries (5 versus 75 m) and isolates (5 versus 70 m) (Table 2). Analysis of variance was not applied to the clades recovered in cultured isolates because the effort to isolate strains was not similar across all depths. These results were not surprising given that the water column was well mixed, particularly in the upper water column (Fig. 1). In contrast to the overlap in the clades isolated from 5 and 70 m, enrichments from 170 m produced strains belonging to clade XVI only, although two *Synechococcus* clades and one *Prochlorococcus* clade were found in the 175-m clone library (Table 2). It is possible that clade XVI strains have a particular adaptation to low-light conditions and were selected under the low-light level (5 to 8 μmol Q m^{-2} s^{-1}) used to incubate the 170-m enrichments. Because neither clone libraries nor culture isolation is completely quantitative, it is not possible to make firm conclusions about the depth distribution of particular clades. However, it is intriguing that some clades are absent from either the surface samples (5 m) (clade XVI) or deep samples (170 or 175 m) (clades I, IV, and VII) (Table 2).

N utilization by isolates. Although most *Synechococcus* strains are capable of growth on nitrate, a few recently isolated strains were reported to be deficient in growth on nitrate (21, 38). In this study, clades I, II, VII, and XVI contained strains isolated using nitrate, suggesting that these clades are not deficient in the utilization of nitrate or nitrite. However, all strains belonging to clade XV were isolated on ammonium. Positive growth on nitrate was observed in the screening of the following strains isolated on ammonium: UW98 and UW109 (clade II) and UW105 and UW140 (clade XVI). In addition,

strains UW76 (clade I) and UW92 (clade VII) were known to grow on nitrate since they were obtained from nitrate enrichments. In contrast, positive growth was not seen for clade XV strains in initial screenings, so we conducted more detailed N utilization experiments on these strains.

A significantly reduced growth rate on nitrate was seen for clade XV strains in comparison to the control clade II strain UW86 (Fig. 4C). When grown under a light-dark cycle, clade XV strains UW69 and UW106 grew at rates of 0.59 ± 0.02 and 0.61 ± 0.17 day^{-1} on ammonium and 0.52 ± 0.08 and 0.57 ± 0.04 day^{-1} on nitrite, respectively. Two growth responses on nitrate were seen for clade XV. The fluorescence either dropped and then increased only after 15 to 20 days (data not shown) or plateaued and then increased slowly (Fig. 4A and B). Cell concentrations determined by flow cytometry for the experiments shown in Fig. 4 mimicked the patterns seen with fluorescence (data not shown). Growth rates of cells grown on ammonium and nitrate differed by a factor of approximately 8 to 17 for UW69 and 6 to 9 for UW106. In comparison, clade II strain UW86 grew on nitrate at rates that differed from that on ammonium by a factor of only 2 (Fig. 4C). After the initial experiment, cultures in nitrate were subsequently transferred again into fresh nitrate medium and monitored for an additional 70 days. Growth rates of UW69 and UW106 in these second transfers remained low (<0.11 day^{-1}), indicating that additional acclimation in nitrate medium did not result in a faster growth rate. We also repeated experiments under constant light, at a higher light level (60 μmol Q m^{-2} s^{-1}), and at a lower temperature to see if these cues might induce a rapid growth response in clade XV on nitrate, but none was seen (data not shown). It is also possible that growth on nitrate is not restricted at the lower concentrations typical of the natural environmental (nanomolar range); however, this was not feasible to test given the sensitivity of the fluorometer.

To test if concentrations of nitrate at 100 μM were inhibitory to cell growth, we performed N recovery experiments on UW69 and UW106 (Fig. 5). Cells acclimated in 100 μM ammonium were transferred into 100 μM nitrate. After 4 or 5 days, the fluorescence plateaued or dropped slightly (Fig. 5), as had been seen in previous experiments (Fig. 4A and B). At this point, either no additional N, 100 μM ammonium, or 100 μM nitrite was added to the tubes (presumably still containing nitrate). Cells grew after the addition of ammonium or nitrite, while cultures given no additional N increased only slightly in fluorescence (Fig. 5). Thus, the presence of 100 μM nitrate in itself does not inhibit cell growth.

In summary, clade XV strains UW69 and UW106 exhibited a markedly reduced growth rate on nitrate in comparison to the growth rate of clade II strain UW86. Because none of the strains tested were axenic, we cannot conclude whether these strains exhibited slow growth because of a reduced efficiency in nitrate utilization or because they cannot utilize nitrate at all and the very slow growth was supported by the recycling of nitrate by heterotrophic bacteria in the culture. In either case, there is a fundamental difference in nitrogen physiology between clade XV strains and the closely related strains in clade II.

Sequencing of *narB*. Because the inability of *Prochlorococcus* ecotypes to grow on nitrate is attributed to the loss of the nitrate reductase gene, *narB* (38, 52), we attempted to PCR amplify a portion of this gene (representing ~78% of the full-length gene in WH 8102) from clade XV to further investigate N utilization in this group. A product of the expected length (~2,000 bp) was successfully amplified and sequenced from four strains, two each from clades II (WH 8012 and UW122) and XV (UW69 and UW106). The 580 predicted

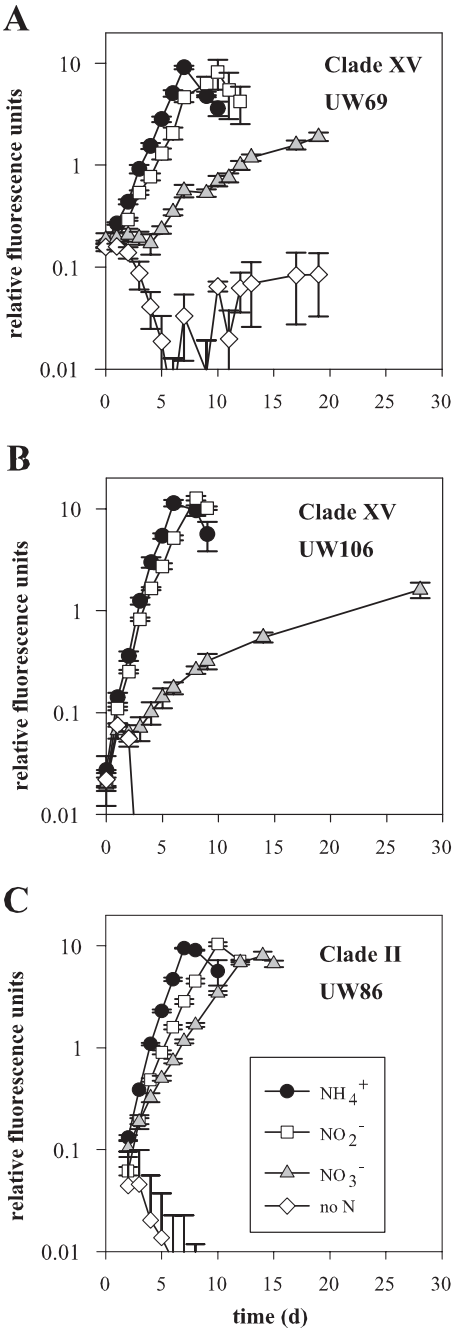

FIG. 4. N utilization experiments for clade XV strains UW69 (A) and UW106 (B) and clade II strain UW86 (C). Cultures were initially acclimated in 100 μM ammonium medium and transferred at time zero into medium containing no N (white diamonds) or 100 μM ammonium (black circles), nitrite (open squares), or nitrate (gray triangles). Symbols represent averages of triplicate tubes, and error bars are standard deviations of triplicates.

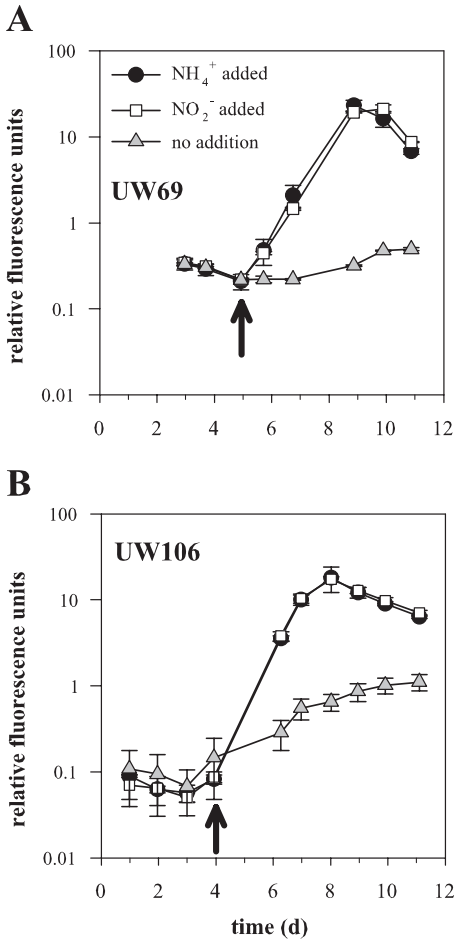

FIG. 5. N utilization recovery experiments for clade XV strains UW69 (A) and UW106 (B). Cultures were initially acclimated in ASW with 100 μM ammonium and transferred at time zero into medium with 100 μM nitrate. Fluorescence units for the initial days are not shown because values were below the detection limit of the fluorometer. After 4 or 5 days (indicated by arrows), cultures were split into three treatments: 100 μM ammonium added (black circles), 100 μM nitrite added (open squares), and no additional N added (gray triangles). Symbols represent averages of triplicate tubes, and error bars are standard deviations of triplicates.

amino acids from usable sequence data for both clade XV strains UW69 and UW106 had high identity to the *narB* genes from clade II strains WH 8012 and UW122 (94% identical) and from the clade III strain WH 8102 (91% identical). No stop codons were found in either of the partial UW69 or UW106 *narB* sequences, and an essential cysteine residue was conserved in both. The topologies of the phylogenetic trees inferred using *narB* and ITS sequences were similar (Fig. 3 and 6). Thus, there is no evidence to suggest that *narB* in strains UW69 and UW106 is not functional. Observed phenotypic differences may be due to differences in the regulation of nitrate reductase or in the capacity to transport nitrate into the cell.

PUB/PEB ratios and chromatic adaptation. Because light adaptation is likely important in ecotype differentiation in the genus marine *Synechococcus*, we assessed the accessory pigment content (PUB and PEB) and control of the PUB/PEB ratio (chromatic adaptation) in selected strains from each ecotype isolated. When grown under white light, *Synechococcus* strains had PUB/PEB ratios ranging from 0.45 to 2.11 (Fig. 7), which falls within the span of previously reported values (21, 51, 64, 68). A large flexibility in the ratio (under white light) was seen within clade II (Fig. 7), and the ratio for clade VII strain UW92 (0.47) (Fig. 7) is considerably lower than values previously reported for other strains within this clade (1.7 to 2.2) (21). This is consistent with previous studies that found that the PUB/PEB ratio is quite flexible within clades (21, 51, 64).

Clade II strain UW90 exhibited an unusual excitation spectrum lacking the distinct peaks near 495 and 545 nm typical of PUB and PEB in other marine *Synechococcus* strains (data not shown). We examined the absorbance spectra of this strain to test for the presence of PUB and PEB. Although a peak characteristic of PUB at 495 nm was present, the expected PEB peak at 545 nm was absent (Fig. 8). Instead, in vivo, UW90 cells exhibited a peak at about 570 nm. Either a modification in the PUB itself or its orientation within the phycobilisome could cause the difference in the absorbance peak for strain UW90. Interestingly, this peak shifted down to ~545 nm in the soni-

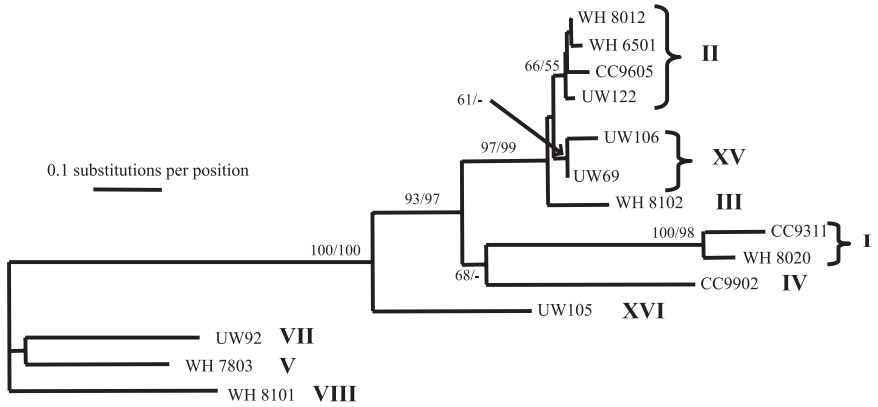

FIG. 6. Phylogenetic relationships of marine *Synechococcus* strains inferred using *narB* sequences. The distance-based tree shown here was inferred using 630 nucleotides with the HKY+G substitution model and with minimum evolution as the criterion (see Materials and Methods). The best inferred tree using the 210 encoded amino acids and distances computed with the Dayhoff PAM matrix had a similar topology (data not shown). Bootstrap values greater than 50 are shown for 1,000 and 100 replicates of nucleotide- or amino acid-based trees, respectively.

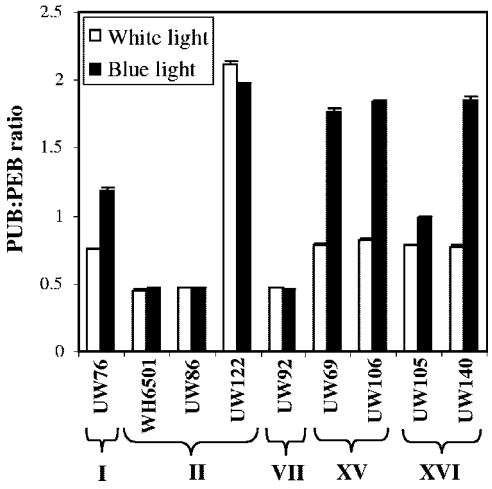

FIG. 7. Ratios of accessory pigments PUB and PEB determined by spectrofluorometry of cultures grown under white (white bars) and blue (black bars) constant light. Bars represent averages of two to four replicate cultures, and error bars represent standard deviations of those replicates. The clade numbers to which the strains belong are given under the strain names.

cated lysate (Fig. 8). The shift could be caused by a disassociation or denaturation of a component of the phycobilisomes.

Strains belonging to clades I, XV, and XVI exhibited chromatic adaptation. These strains had significantly higher PUB/PEB ratios when grown under blue light than when grown under white light (Fig. 7). Clade I strain UW76 is identical in ITS sequence to strain WH 8020, which has been previously shown to be chromatically adaptive (43). In contrast, chromatic adaptation was not observed in strains belonging to clades II and VII (Fig. 7). The presence of chromatic adaptation in clade XV and not in clade II is a second aspect in which clade XV is physiologically distinct from clade II. The unique absorbance spectra of strain UW90, variable PUB/PEB ratios within putative ecotypes, and variability in the magnitude of the chromatic adaptation response within clade XVI (Fig. 7) demonstrate that light physiology can be quite flexible even within ecotypes.

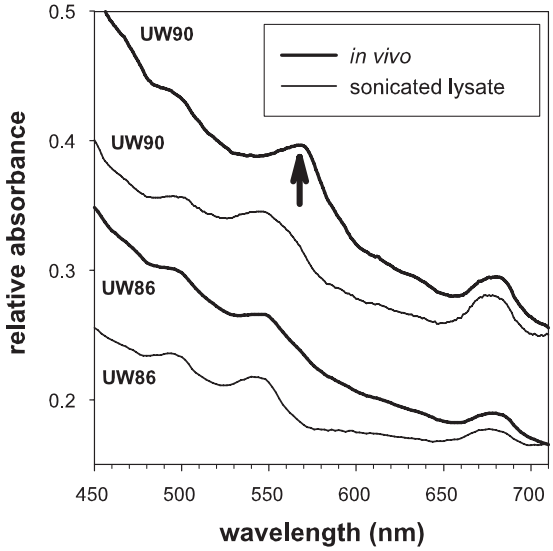

FIG. 8. Absorbance spectra of in vivo cells (thick lines) or sonicated cell lysates (thin lines) for clade II strains UW86 and UW90 grown under constant white light. UW86 exhibits a peak at 545 nm, which is typical for marine *Synechococcus* strains and represents the accessory pigment PEB. Note that this peak is seen for strain UW90 lysate, but it is shifted to 570 nm in the spectrum of in vivo cells (arrow).

DISCUSSION

Using a combination of culturing and culture-independent approaches, seven distinct *Synechococcus* phylotypes were recovered from a well-mixed water column from the Sargasso Sea during spring, a time when *Synechococcus* cell numbers are at their annual peak (13). Previous surveys of diversity in various coastal and oligotrophic habitats consistently reported what we have found here, that *Synechococcus* communities are composed not of a single genotype but of many distinct genetic clades that are believed to represent physiologically and genetically distinct populations or ecotypes (18, 21, 30, 39, 54, 62, 64). Unfortunately, it is not possible to compare the diversity recovered here with those of other studies because of differences in the coverage of clone library sequencing. Although there was overlap in the clades found by either method, culture-dependent and -independent methods sampled different portions of the community, as has been observed with other microbial systems (14, 55, 58). Our use of both methods in tandem gave a more complete picture of the natural diversity; however, the number of ecotypes found still likely represents the minimum value for the total diversity in situ. Some *Synechococcus* ecotypes represent less than 1% of the total *Synechococcus* community (22); therefore, more sensitive methods such as real-time PCR may be needed to detect such rare types (2, 73). The sequences obtained in this study will be valuable for the development of molecular assays such as real-time PCR to explore *Synechococcus* community structure and ecology.

We have isolated two new phylogenetic clades of marine *Synechococcus* strains, labeled clades XV and XVI, and we posit that they represent ecologically distinct populations or ecotypes. Identifying how phylogenetic clades differ physiologically is essential to a further understanding of the ecology of these communities. Delineation of phylogenetic clades is often done subjectively, and unfortunately, there is no consensus on a species concept for prokaryotes, nor is there an objective means for delineating clades of sequences (24). Universal sequence identity cutoffs are not appropriate, particularly since rates of evolution vary between genes, between diverse bacterial lineages, and even between closely related taxa (40, 71). Clade groupings using both ITS and *narB* sequences are consistent with the empirically observed pattern that the average distance between ecologically distinct clades is generally at least twice the average distance within each clade (46), but this criterion is based on taxa distantly related to *Synechococcus* and may not hold as a general rule for all bacteria. There clearly is a need to further develop objective methods for identifying putative ecotypes based on sequence information. Multilocus sequence typing is one such approach (24). Another emerging approach is to examine changes in the number of sequence clusters found while varying the percent identity

threshold, but more work is required to understand the behavior of curves generated by this method and the conclusions that can be drawn from them (1).

Ultimately, physiological evidence is needed to decisively establish phylogenetic clades as ecologically distinct. We looked at light and N physiology to further establish clades XV and XVI as ecotypes and to investigate the modes of ecotype differentiation in marine *Synechococcus* strains. Strains from both clade XV and XVI are capable of chromatic adaptation, revealing that this trait is more widely distributed among marine *Synechococcus* strains than previously known. Both chromatic adaptation and a reduced efficiency in nitrate utilization establish clade XV as physiologically distinct from clade II, its closest relative; thus, we putatively designate this clade as a distinct ecotype. Similarly, we conclude that clade XVI is a putative ecotype, given its large divergence from other clades and because it possesses chromatic adaptation, while its most closely related clade (VII) does not. Although further testing is required to discover the mechanism behind the reduced efficiency of nitrate utilization in clade XV, it is likely that this deficiency excluded this clade from previous isolation efforts, most of which have been done with nitrate as the sole N source. Our study confirms that despite previous biases in isolation, most marine *Synechococcus* strains appear to be capable of nitrate utilization (21, 38).

The two main mechanisms of evolution that cause phenotypic changes are changes in gene content (by loss or acquisition of genes) and the modification of existing genes. The former mechanism is known to be important in the differentiation of marine cyanobacteria, and in particular, the absence of *narB* causes the deficiency in nitrate utilization in *Prochlorococcus* (12, 45, 52); however, the genomic changes that shape phenotypic differences between ecotypes can be more complex than simply the loss or acquisition of a single functional protein. This may be the case for clade XV strains. Although they appear to have a functional copy of *narB*, the loss or modification of other genes involved in nitrate utilization, such as N regulation genes or a nitrate transporter, could cause the reduced growth efficiency on nitrate. The gradual evolution of existing capabilities rather than the loss of whole metabolic pathways has been implicated in two studies of heterotrophic bacteria in which distinct taxa are delineated not just by the capacity to use particular organic substrates but also by differences in growth rates on some compounds upon which they can all grow (15, 36). The relative importance to ecotype differentiation of such gradual evolution resulting in quantitative phenotypic differences versus the complete loss or gain of particular physiologies is not well studied (15).

Many aspects of light physiology are quite flexible within *Synechococcus* ecotypes. Specifically, the PUB/PEB ratio varies within clades II, III, and VII (51, 64; this study); strain UW90 exhibits an absorption spectrum distinct from those of other members of its clade, and the magnitude of the PUB/PEB ratio change varies between strains of chromatically adaptive clade XVI. Although chromatic adaptation appears to be a cohesive characteristic of some ecotypes (clades I, XV, and XVI), chromatic adaptation is not monophyletic within clade III (43). One explanation for such physiological diversity is that it represents distinguishing traits of still more ecologically distinct populations and thus that we have set our ecotype designations too broadly. Alternatively, for the marine bacterium *Vibrio splendidus*, it has been postulated that similar genomic variability (and assumed correlated physiological diversity) within natural populations represents diversity within a single ecologically distinct population, which, although not neutral, is selected for only under ephemeral conditions and which thus does not become fixed in the population (61). This second explanation may apply to the variability in light physiology seen within *Synechococcus* ecotypes, since current phylogenetic data do not support further division for these ecotypes. It is also possible that the N utilization deficiency that we observed in clade XV strains is an example of such physiological flexibility within an ecotype. Physiological characterization of additional clade XV strains, some of which have recently been isolated from the Sargasso Sea (I. Ehrenreich and E. Webb, personal communication), could help address this question.

An understanding of the evolutionary processes leading to ecotype differentiation in marine *Synechococcus* strains will help explain the mechanism by which these closely related microbial clades coexist in the natural environment. The coexistence of multiple ecotypes requires adaptation to distinct niches, which can be driven by heterogeneity in habitat with both space and time (26). In a stratified water column, gradients in parameters such as light and nutrients can create distinct habitats. Indeed, *Prochlorococcus* ecotypes clearly partition the water column with depth according to light adaptation (2, 52, 70, 73), and there is some evidence that *Synechococcus* clade II may be more dominant in surface waters (63). However, here, we observed at least seven marine *Synechococcus* ecotypes coexisting in a well-mixed water column. A diverse array of niches could be supported by physical heterogeneity at the scale of micropatches (5, 34) or through temporal variability. Ecotypes may have seasonal successions as do larger classes of picophytoplankton (13, 31) such that the particular ecotypes that we have observed at this time point may reflect a *Synechococcus* community in the midst of a transition as niche availability changes with time. Interactions between microbes may create complexity and provide another mechanism to support the coexistence of several ecotypes, as has been documented in the laboratory for *Escherichia coli* and *Pseudomonas fluorescens* (27, 49). Here, multiple *Synechococcus* ecotypes were commonly found to occur in enrichment cultures over a time period of at least 2.5 months (estimated to be at least 35 generations). In natural communities, the potential importance of ecotype interactions in maintaining diversity is largely unexplored.

In summary, we report the assessment of marine *Synechococcus* diversity within a well-mixed water column and the discovery of two new putative ecotypes distinguished by their light physiology and nitrogen utilization capabilities. Clearly, interpreting the significance of phylogenetic and physiological diversity in natural bacterial populations is an ongoing challenge, and there is still much to learn about how the processes of selection and genetic drift result in the fixation of some traits within an ecotype while maintaining diversity in others. The development of high-throughput molecular assays to quantify the abundance of *Synechococcus* ecotypes in time and space will allow us to understand how physiological variability impacts the structure of natural communities.

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