APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1999, p. 5247–5251 Vol. 65, No. 12
0099-2240/99/$04.00+0

Swimming Marine *Synechococcus* Strains with Widely Different Photosynthetic Pigment Ratios Form a Monophyletic Group

GERARDO TOLEDO, B. PALENIK, AND B. BRAHAMSHA*

Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093-0202

Received 14 July 1999/Accepted 10 September 1999

Unicellular marine cyanobacteria are ubiquitous in both coastal and oligotrophic regimes. The contribution of these organisms to primary production and nutrient cycling is substantial on a global scale. Natural populations of marine *Synechococcus* strains include multiple genetic lineages, but the link, if any, between unique phenotypic traits and specific genetic groups is still not understood. We studied the genetic diversity (as determined by the DNA-dependent RNA polymerase *rpoC1* gene sequence) of a set of marine *Synechococcus* isolates that are able to swim. Our results show that these isolates form a monophyletic group. This finding represents the first example of correspondence between a physiological trait and a phylogenetic group in marine *Synechococcus*. In contrast, the phycourobilin (PUB)/phycoerythrobilin (PEB) pigment ratios of members of the motile clade varied considerably. An isolate obtained from the California Current (strain CC9703) displayed a pigment signature identical to that of nonmotile strain WH7803, which is considered a model for low-PUB/PEB-ratio strains, whereas several motile strains had higher PUB/PEB ratios than strain WH8103, which is considered a model for high-PUB/PEB-ratio strains. These findings indicate that the PUB/PEB pigment ratio is not a useful characteristic for defining phylogenetic groups of marine *Synechococcus* strains.

Unicellular marine cyanobacteria are abundant in both coastal and oligotrophic environments (6, 19, 21), where they contribute substantially to primary production (15). Phylogenetic analyses of isolates have shown that the *Synechococcus* group comprises multiple genetic lineages (9, 25, 30, 31, 39). These lineages have also been identified in situ, as demonstrated with shotgun-cloned *rpoC1* libraries (11, 23). The relationship, if any, between specific genetic lineages and unique physiological adaptations is not currently understood.

Marine *Synechococcus* strains differ from each other physiologically in a number of ways. The differences include differences in the responses to nitrogen depletion (14), in preferences for nitrate or urea for growth (8, 26, 33), in cell size and growth rate (26, 33), and in cell cycle behavior (1).

There is also physiological diversity in the *Synechococcus* light-harvesting apparatus. *Synechococcus* cells harvest light with a structure known as the phycobilisome (13). In open-ocean *Synechococcus* strains phycoerythrin (PE) is the primary light-harvesting protein in the phycobilisome. There are two PE proteins encoded by different genes in marine *Synechococcus* strains, and they absorb light with the covalently attached chromophores phycourobilin (PUB) (maximum absorption at 495 nm) and phycoerythrobilin (PEB) (maximum absorption at 545 nm) (22). The in vivo absorption properties of cells are due mostly to the PUB and PEB chromophores and to chlorophyll *a*. It is thought that the presence of these two PE proteins in marine strains reflects evolutionary chromatic adaptation since these proteins enhance light harvesting at the blue wavelengths which penetrate the water column in oceanic waters (37).

Cultivated marine *Synechococcus* strains exhibit different PUB/PEB chromophore ratios (33), and the strain-specific differences can be relatively constant under different growth conditions (21, 38). Based on this observation, when multiple pigment signatures have been observed in the environment by using flow cytometry or fluorescence spectra, it has been proposed that they result from genetically distinct populations (17, 20, 21, 38, 40).

Another example of physiological diversity in the genus *Synechococcus* is the ability of some marine strains to swim by means of a unique type of motility that is characterized by the absence of flagella or any other visible motility organelle (35). It is likely that motility is an ecologically relevant trait, especially in the open ocean. Motile strains exhibit chemotactic responses to nitrogenous compounds, such as ammonia, nitrate, urea, glycine, and β-alanine, at concentrations relevant in the oligotrophic oceans (36). Hence, as an adaptive trait, motility may result in an enhanced ability to find and take up nitrogen, often a likely limiting resource in marine systems, as well as access to unique ecological niches, such as the microenvironments around particles and larger phytoplankton cells (36).

The motile marine *Synechococcus* strains currently in culture display a range of PUB/PEB chromophore ratios (33). In characterizing motile isolates from the California Current, we realized that this range was even broader and encompassed ratios exhibited by some nonmotile strains. As part of ongoing efforts to understand the spatial and temporal structure of the California Current cyanobacterial community, we investigated the link between two physiological characteristics, motility and pigment complement, and phylogeny. To do this, the phylogenetic diversity of motile *Synechococcus* strains and members of other unicellular cyanobacterial lineages was determined by using DNA-dependent RNA polymerase gene sequences. Furthermore, the PUB/PEB ratios of motile strains, including the ratios of several new isolates, were determined. We show here that motile marine *Synechococcus* strains form a monophyletic group whose members exhibit a wide range of PUB/PEB ratios; our data indicate that motility is a better marker of genetic affiliation than pigment ratios are.

* Corresponding author. Mailing address: Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0202. Phone: (858) 534-7505. Fax: (858) 534-7313. E-mail: bbrahamsha@ucsd.edu.

TABLE 1. *Synechococcus* strains used in this study and some of their characteristics

Strain	Geographic region of isolation	PUB/PEB ratio[a]	Source	Motility
E. Lake	Freshwater (Yosemite National Park)	No PUB	B. Palenik[b]	−
WH7803	Atlantic Ocean	0.4	J. Waterbury[c]	−
WH7805	Atlantic Ocean	No PUB	J. Waterbury[c]	−
WH8011	Atlantic Ocean	0.8	J. Waterbury[c]	+
WH8102	Atlantic Ocean	2.0	J. Waterbury[c]	+
WH8103	Atlantic Ocean	1.2	J. Waterbury[c]	+
WH8112	Atlantic Ocean	1.1	J. Waterbury[c]	+
WH8113	Atlantic Ocean	1.0	J. Waterbury[c]	+
CC9301[d]	California Current	2.0	B. Brahamsha	+
CC9702	California Current	1.6	This study	+
CC9703	California Current	0.4	This study	+
C129	Red Sea	0.9	A. Post and D. Lindell	+

[a] Ratio of the peaks observed in the fluorescence excitation spectra when emission was measured at 570 nm. These peaks corresponded to PUB and PEB peaks at approximately 495 and 545 nm, respectively.
[b] See reference 25.
[c] See reference 33.
[d] This strain has been designated mot2 previously (30).

MATERIALS AND METHODS

Strain isolation and growth conditions. The strains used in this work and some of their relevant properties are shown in Table 1. Strains CC9702 and CC9703 were isolated from the oligotrophic edge of the California Current at stations 93.120 (30 m) and 77.100 (70 m), respectively, of the CalCOFI (California Cooperative Oceanic Fisheries Investigations, cruise 9709) grid pattern. The isolation procedure used was the procedure described by Toledo and Palenik (30), with the following modification: the original inoculum was either filtered through a 1.2-μm-pore-size filter or not filtered. The unfiltered treatment resulted in enrichment cultures containing eukaryotic cells and unicellular cyanobacteria. After dense cyanobacterial growth was observed, the samples were filtered through a 1.2-μm-pore-size filter to remove large eukaryotic cells and to obtain a cyanobacterial enrichment culture; this was followed by plating. Clonal isolates were obtained by two rounds of pour plating (3) and isolation of single colonies. All strains were grown in SN medium (34) at a light intensity of 0.15×10^{16} quanta m^{-2} s^{-1} under cool white fluorescent tubes at 23°C. Freshwater *Synechococcus* sp. strain E. Lake was grown in BG11 medium (7) containing 1.7 mM $NaNO_3$.

Motility was assessed by phase-contrast microscopy by using wet mounts of logarithmic- and stationary-phase cultures. Motile isolates also exhibited diffuse colony morphology in pour plates, as reported previously by Brahamsha (3).

DNA extraction and PCR amplification. Genomic DNA was extracted as described by Brahamsha (3) and was purified further with a Gene Clean kit (Bio 101). Alternatively, DNA was extracted from cells by using a High Pure PCR template preparation kit (Boehringer Mannheim) as recommended by the manufacturer. The PCR primers used and the method used to amplify the 612-bp *rpoC1* gene fragment have been described previously (24, 30). PCR performed with DNA from motile strains sometimes resulted in low yields of the 612-bp fragment and often a smaller PCR product. Based on its sequence, the latter product is not related to *rpoC1* (data not shown). The amplified band whose size corresponded to the size of *rpoC1* was then excised from the agarose gel, purified, cloned, and sequenced as described below. DNA was purified from the agarose gel with a Gene Clean glassmilk purification kit (Bio 101).

Cloning and sequencing. The products of PCR were cloned either directly or after gel purification into the pCR2.1 cloning vector (TA cloning kit or TA-topo kit; Invitrogen) by following the manufacturer's instructions. The nucleotide sequences of both strands of the cloned 612-bp *rpoC1* fragment were determined by automated sequencing of plasmid templates performed with a PRISM Ready Reaction DyeDeoxy terminator cyle sequencing kit and a model 373 DNA sequencer (Applied Biosystems).

Phylogenetic analysis. Neighbor-joining trees were constructed with Jukes-Cantor corrected distances. A bootstrap analysis was performed with 100 subsamples. All of the programs used for analysis were obtained from the PHYLIP package, version 3.572 (10). Strains CC9616, CC9617, CC9701, CC9605, and SS9401 are nonmotile marine *Synechococcus* spp. strains that were isolated from either the California Current (CC9616, CC9617, CC9701, and CC9605) or the Sargasso Sea (SS9401) and will be described elsewhere (26).

Pigment analysis. In vivo absorption spectra for strains WH7803, WH8103, CC9702, and CC9703 were obtained by using cultures in the logarithmic growth phase. The spectral absorption coefficients of cell suspensions were determined in 1-cm quartz cuvettes with a dual-beam spectrophotometer (model Cary 100E; Varian) equipped with an integrating sphere (model DRA-CA-30; Labsphere). Measurements were made in the spectral region from 400 to 750 nm at a resolution of 1 nm, and sterile SN medium was used to determine the instrument baseline and as the reference. Individual spectra were smoothed with a 5-nm moving average, and the spectral absorption coefficient at 750 nm was subtracted from the entire spectrum to correct for scattering. The mean cellular absorption cross-section, $\bar{\sigma}_a$ (28), was calculated by normalizing the spectral absorption coefficient to the number of cells per unit of suspension volume. Cells were enumerated with a FACSort flow cytometer (Becton Dickinson) equipped with an air-cooled laser providing 15 mW at 488 nm. A 5-μl aliquot of each culture was diluted in 990 μl of sterile phosphate-buffered saline (pH 7.5), and 5 μl of green fluorescent microbeads (diameter, 0.9 μm; Duke Scientific) was added as an internal reference. Cell counts were standardized to the volume of the sample analyzed by the instrument, as estimated from the running time.

Fluorescence excitation spectra were obtained with whole-cell suspensions by using a Fluoromax 2 spectrofluorometer (ISA Instruments). Emission was measured at 570 nm. The excitation slit was 1 nm, while the emission slit was 5 nm. Spectra were obtained in the ratio mode. The PUB/PEB ratio was calculated by determining the ratio of relative fluorescence from the PUB peak (excitation around 492 nm) to relative fluorescence from the PEB peak (excitation around 545 nm). The actual peaks were used rather than specific wavelengths. This ratio estimated the PUB/PEB ratio in PE since presumably excitation of PUB or PEB chromophores (sometimes part of phycocyanin) would result in fluorescence emission from the terminal energy acceptor in phycocyanin, a phycocyanobilin chromophore (29). This would occur at 644 nm, a wavelength significantly longer than 570 nm. When no PUB peak was present (strains WH7805 and E. Lake), the result was indicated as No PUB.

Nucleotide sequence accession numbers. DNA sequences of the amplified 612-bp *rpoC1* fragment from *Synechococcus* spp. motile strains CC9301, WH8112, WH8011, WH8113, WH8102, WH9702, WH9703, and C129 have been deposited in the GenBank database under accession no. AF153332 to AF153339, respectively. The nucleotide sequence accession numbers for strains CC9605, CC9616, CC9617, and SS9401 are AF154560 to AF154563, respectively, while the nucleotide sequence accession number for strain CC9701 is AF155131. Accession numbers for the other strains used in this study have been published previously (24, 25, 30).

RESULTS

Two new motile *Synechococcus* strains, designated strains CC9702 and CC9703, were isolated from the California Current. During our characterization of these isolates, we observed that they had noticeably different pigment characteristics; cultures of CC9702 were brown, while cultures of CC9703 were pink (data not shown), suggesting that the PUB/PEB ratios of the two strains were different. This was confirmed by comparing the in vivo absorption spectra of the strains (Fig. 1), as well as their fluorescence excitation spectra (Fig. 2). The absorption spectrum of strain CC9703 was remarkably similar to that of nonmotile isolate WH7803 (Fig. 1), a model representative of strains that have low PUB/PEB ratios (group II [33]), while the absorption spectrum of CC9702 was more similar to that of

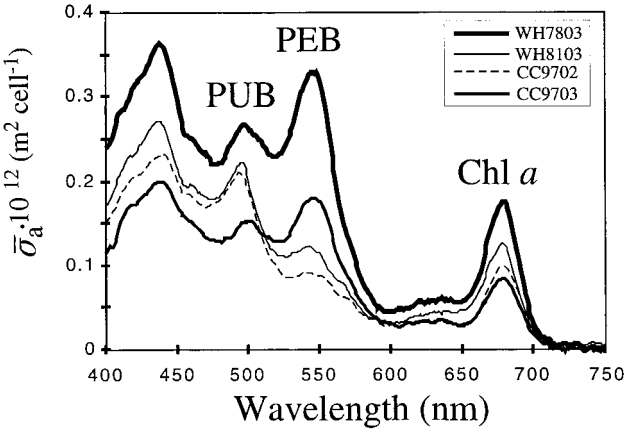

FIG. 1. In vivo spectral absorption cross-section for *Synechococcus* sp. strains WH7803, WH8103, CC9702, and CC9703. The peaks corresponding to PUB, PEB, and chlorophyll *a* (Chl *a*) are indicated.

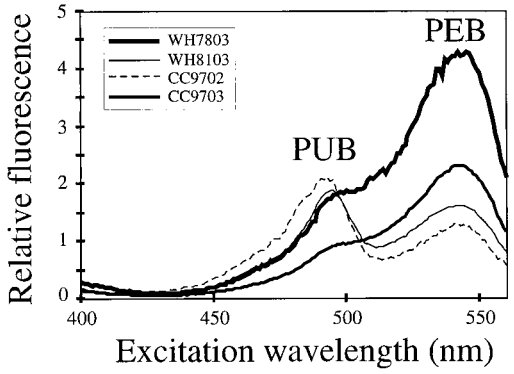

FIG. 2. In vivo fluorescence excitation spectra of *Synechococcus* sp. strains WH7803, WH8103, CC9702, and CC9703. Emission was measured at 570 nm. The peaks corresponding to PUB and PEB are indicated.

motile strain WH8103, which is considered a model organism for the high-PUB/PEB-ratio group (group V [33]) (Fig. 1).

In order to examine pigment diversity in the motile strains, we obtained the fluorescence excitation spectra of nine motile strains and determined the PUB/PEB ratios (Table 1). We obtained a wide range of PUB/PEB chromophore ratios for the strains analyzed. It appears that the PUB/PEB ratios of the motile strains that we examined are diverse and do not fit into discrete high- and low-ratio groups. The range encompasses the values obtained for WH8103 and for WH7803.

rpoC1 gene fragments were amplified from nine motile strains (Table 1). A phylogenetic analysis of the *rpoC1* gene sequences of motile *Synechococcus* strains showed that these organisms belong to a single lineage that is independent of all other marine or freshwater groups (Fig. 3). Our phylogenetic analyses were robust, as shown by the high bootstrap values at each node based on 100 replicates. There was some genetic diversity within the group, as all WH motile isolates and strains CC9702 and CC9301 were closely related (99% identity at the nucleotide level), while strains CC9703 and C129 were more divergent (Fig. 3) and exhibited 96% identity at the nucleotide level with the members of the former group. A comparison with 40 environmental cloned sequences detected in shotgun clone libraries revealed that there are at least two closely related clones (MLG5 and MLG30) obtained from the California Current (11) that have not been cultivated yet but potentially have a motile phenotype. These environmental clones and all motile strains contain two unique amino acid residues that are potentially diagnostic among *Synechococcus* spp., a valine and a serine at positions 199 and 202 of the 204-amino-acid fragment (data not shown).

DISCUSSION

Natural populations of marine *Synechococcus* include multiple genetic lineages, but the link, if any, between unique physiological traits and genetic groups is not well understood. In this work, we found that marine *Synechococcus* strains that share the unique property of nonflagellar swimming form a monophyletic group despite differences in pigment characteristics. Although we do not understand the mechanism of swimming in *Synechococcus* spp. yet, it, like diverse other forms of prokaryotic motility (16, 18), is likely to require numerous genes and gene products (2, 7). Such putative complexity may account for the fact that nonflagellar swimming appears to have arisen only once in marine *Synechococcus* spp.

Examples (such as the one presented here) in which a phylogenetically coherent group of cyanobacteria can be defined by a physiological characteristic are rare. Recently, Garcia-Pichel et al. (12) showed that a group of extremely halotolerant cyanobacteria, the Halothece cluster, is monophyletic. Within this group there is a great deal of morphological and physiological diversity. Garcia-Pichel et al. suggested that gaining access to the hypersaline niche allowed the ancestor of the Halothece group to evolve adaptations suited to more specific niches within that environment. Similarly, the physiological diversity in pigment types among motile *Synechococcus* strains may stem from adaptations of motile populations to environmental niches with different light intensities, spectral compositions, or nutrient (especially nitrogen) regimes, which resulted in selection for different PUB/PEB ratios. It has been proposed that PE plays a role in nitrogen storage (41), and it is possible that in addition to affecting light-harvesting properties, chromophore diversity may be linked to differences in nitrogen metabolism and storage.

Motile marine *Synechococcus* strains are not usually found in nutrient-rich coastal regions, but they are found in oligotrophic regions of the world's oceans where nitrogen is often limiting (20, 33). While motile *Synechococcus* strains appear to exhibit neither phototactic nor photophobic responses, Willey and Waterbury (36) have shown that at least one motile *Synechococcus* strain (strain WH8113) is chemotactic toward a variety of nitrogenous compounds at ecologically relevant concentrations. These researchers suggested that for organisms

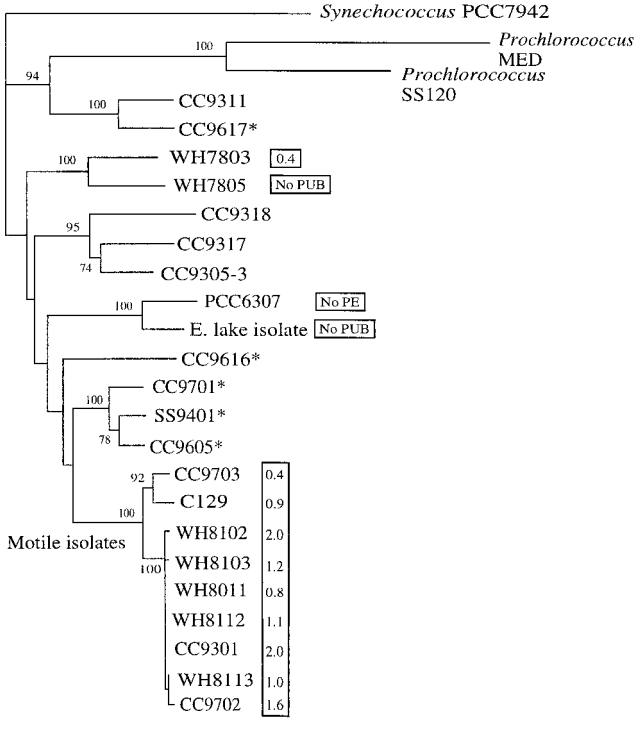

FIG. 3. Neighbor-joining tree constructed with Jukes-Cantor corrected distances by using a 612-bp fragment of the DNA-dependent RNA polymerase (*rpoC1*) gene sequence of different *Synechococcus*-like isolates. Bootstrap values based on 100 replicates are shown at each node. Scale bar = 0.1 nucleotide substitution per site. The values in boxes are PUB/PEB fluorescence ratios. Asterisks indicate strains whose sequences were obtained from reference 26.

living in oligotrophic environments, the ability to detect and seek out patches of nutrient enrichment, such as larger phytoplankton cells or microaggregates (marine snow), may provide an ecological advantage. Hence, chemotaxis toward particles or other cells exuding nitrogenous compounds may provide a unique niche for motile *Synechococcus* strains compared to nonmotile strains that share the same environment and have the same pigment composition.

In order to determine whether motility is correlated with particular physical and chemical properties of the water column, such as nitrogen content or aggregate abundance, it will be necessary to understand the distribution of motile *Synechococcus* strains. On the basis of the number of isolates obtained from enrichment cultures (20, 33), motile *Synechococcus* strains have been reported to be numerous, especially in the open ocean. However, very little is known about the in situ abundance of these organisms relative to the abundance of nonmotile strains. An examination of environmental libraries has indicated that clones that group with motile strains do not appear to be well-represented (11). It is possible that this is due to the poor efficiency of *rpoC1* amplification from motile strains with the standard primers.

The approaches used for in situ enumeration of cyanobacteria have included immunofluorescence, which has been used successfully to enumerate certain PE-containing *Synechococcus* serogroups in field samples (5), and, more recently, fluorescence in situ hybridization (27). A direct assay for motile strains currently being developed utilizes an antibody specific for SwmA (2), an abundant cell surface polypeptide that is required for swimming in *Synechococcus* spp. This antiserum reacts with all of the motile strains examined to date but not with nonmotile strains (4) and hence should be useful for assessing the distribution of motile strains in field samples.

Finally, the data presented here indicate that pigment characteristics, particularly PUB/PEB ratios, are not useful for delineating *Synechococcus* phylogenetic groups. One of the principal characteristics used to define marine *Synechococcus* groups has been the PUB/PEB ratio (33). While some phylogenetic analyses have demonstrated that there is a correspondence between pigment groups and specific lineages (9, 30, 38), these analyses were based on a limited number of isolates. We show here that there is pigment diversity within a unique lineage, the motile *Synechococcus* lineage. The PUB/PEB ratios of nine motile strains were very diverse, spanning the known range of values obtained for marine *Synechococcus* strains. Such pigment diversity was also observed with two nonmotile *Synechococcus* clusters, a cluster consisting of marine strains WH7803 and WH7805 and a cluster consisting of freshwater strains PCC6307 (32) and E. Lake (Fig. 3). We may eventually learn that only some clades exhibit pigment diversity or that all clades exhibit pigment diversity.

ACKNOWLEDGMENTS

This research was supported by NSF grant OCE9633111. G.T. was partially supported by CONACYT grant 92755.

We thank Rick Reynolds for help with the cellular absorption cross-section analysis; Greg Mitchell and Vic Vacquier for the use of the integrating sphere spectrophotometer and the spectrofluorometer, respectively; and Anton Post and Debbie Lindell for providing *Synechococcus* sp. strain C129.

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