APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1997, p. 4298–4303 Vol. 63, No. 11
0099-2240/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Synechococcus Diversity in the California Current as Seen by RNA Polymerase (*rpoC1*) Gene Sequences of Isolated Strains

GERARDO TOLEDO AND BRIAN PALENIK*

Marine Biology Research Division and Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093-0202

Received 17 July 1997/Accepted 8 September 1997

Because they are ubiquitous in a range of aquatic environments and culture methods are relatively advanced, cyanobacteria may be useful models for understanding the extent of evolutionary adaptation of prokaryotes in general to environmental gradients. The roles of environmental variables such as light and nutrients in influencing cyanobacterial genetic diversity are still poorly characterized, however. In this study, a total of 15 *Synechococcus* strains were isolated from the oligotrophic edge of the California Current from two depths (5 and 95 m) with large differences in light intensity, light quality, and nutrient concentrations. RNA polymerase gene (*rpoC1*) fragment sequences of the strains revealed two major genetic lineages, distinct from other marine or freshwater cyanobacterial isolates or groups seen in shotgun-cloned sequences from the oligotrophic Atlantic Ocean. The California Current low-phycourobilin (CCLPUB) group represented by six isolates in a single lineage was less diverse than the California Current high-phycourobilin (CCHPUB) group with nine isolates in three relatively divergent lineages. The former was found to be the closest known genetic group to *Prochlorococcus* spp., a chlorophyll *b*-containing cyanobacterial group. Having an isolate from this group will be valuable for looking at the molecular changes necessary for the transition from the use of phycobiliproteins to chlorophyll *b* as light-harvesting pigments. Both of the CCHPUB and CCLPUB groups included strains obtained from surface (5 m) and deep (95 m) samples. Thus, contrary to expectations, there was no clear correlation between sampling depth and isolation of genetic groups, despite the large environmental gradients present. To our knowledge, this is the first demonstration with isolates that genetically divergent *Synechococcus* groups coexist in the same seawater sample.

Cyanobacteria of the genus *Synechococcus* are major contributors to the primary productivity of ocean and freshwater ecosystems. The diversity of this genus has been evaluated by using morphological traits and moles percent G+C content of strains in culture (27), proportions of pigments, and other physiological characteristics (28), and more recently, a number of molecular genetics tools have been applied to characterize *Synechococcus* and cyanobacterial diversity, such as restriction fragment length polymorphism analysis (5, 33), 16S rRNA (11, 25, 31), and DNA-dependent RNA polymerase gene (*rpoC1*) fragment sequences (20).

One of the core subunits of RNA polymerase is present in cyanobacteria as two subunits encoded by the *rpoC1* and *rpoC2* genes, which are present as single copies in cyanobacterial genomes (1). The evolutionary origins of this difference in subunit structure are unknown, but may be due to a split in an early core subunit (18). *rpoC1* sequences can be easily and specifically PCR amplified from cyanobacteria and used to elucidate phylogenetic relationships between distantly related cyanobacterial groups, especially when the third codon position is eliminated from the data set (18, 21). Phylogenetic trees derived with *rpoC1* data match those derived with 16S rRNA data. When the third codon position is included, *rpoC1* shows greater divergence between two related strains and is thus better able to resolve genus-species-level questions than 16S rRNA. For example, the 16S rRNA gene differs by 1.4% between *Synechococcus* sp. strain WH7805 and strain WH8103, while the 612-bp *rpoC1* fragment differs by 17%. Apparently closely related but distinct cyanobacterial groups were seen by *rpoC1* analysis of a DNA sample from the open ocean (19). In general, the use of RNA polymerase gene sequences as a phylogenetic tool is increasing in other taxonomic groups as well (16).

Environmental clines have a strong influence on *Synechococcus* diversity in several environments. In hot spring microbial mats, temperature is a major determinant of genetic groups (9). In Lake Constance, a freshwater lake, a highly diverse population of *Synechococcus* strains can be seen by restriction fragment length polymorphism analysis, and the relative abundance of these strains may be changing as a function of season and nutrient status of the lake (7, 22).

In the oligotrophic ocean environment covering much of the globe, large changes in some environmental parameters occur with depth. For example in the oligotrophic regions of the California Current (6), light flux drops by 3 orders of magnitude from the surface to the bottom of the cyanobacterial growth range (0 to 150 m). Large shifts in light quality occur from full sunlight to the blue wavelengths (475 to 500 nm) predominating at depth. Nutrient gradients are also large, with a range of nitrate concentrations from undetectable through much of the upper water column to detectable at a few micromolar units at depth. Temperature gradients are smaller with the range in the California Current from around 20°C at the surface to about 13°C at the bottom of the euphotic zone. This picture is then complicated by a seasonal mixing cycle. During the winter, the water column is mixed through the euphotic zone, while during summer and fall, the water column is generally stratified. During stratification, different parts of the cyanobacterial population are presumably under very different selection pressures with high-light, low nutrient conditions at

* Corresponding author. Phone: (619) 534-7505. Fax: (619) 534-7313. E-mail: bpalenik@ucsd.edu.

the surface and lower, blue light and higher nitrate conditions at depth. We would expect the most genetic differentiation in the cyanobacterial population thus between surface and deep samples.

Marine *Synechococcus* isolates often contain the light-harvesting protein phycoerythrin, but with various ratios of the chromophores phycourobilin (PUB) and phycoerythrobilin (PEB) in the protein. The ratios can be genetically or physiologically set (28, 32). Olson et al. (17) have demonstrated that cells with apparent low PUB levels were more prevalent near shore and in surface waters of the California Current and other coastal areas, while cells with high PUB levels are found deeper in the water column. These results could be explained as the advantage of the pigment PUB over PEB for absorbing blue light. They also found that multiple populations could coexist in some samples. The presence of multiple populations seen by flow cytometry, however, is not evidence for genetic differentiation, because it could be due to the presence of photoadaptation, mixing, or nutrient effects on a single group.

From studies of the depth distributions of eukaryotic phytoplankton, it has been found that different morphological species are characteristic of surface versus deep environments (26), but similar studies have not been done with marine cyanobacteria. The aim of this study was to examine genetic differentiation in the cyanobacteria (especially within *Synechococcus*-like strains) as a function of depth with the expectation that surface strains would be evolutionarily adapted to surface conditions and thus genetically different from deeper isolates. This hypothesis could be tested in several ways. One method is a shotgun cloning approach, in which the population is sampled by amplification and sequencing of a gene or gene fragment from a DNA sample. 16S rRNA has been used in this way to look at the depth distribution of prokaryotic groups at higher taxonomic levels, for example (10). This approach is also starting to be used with *rpoC1* sequencing (19). The PCR approach has potential problems in that there may be PCR amplification biases and the resulting data provide a picture of diversity but do not provide much understanding of why that diversity is present. Without an isolate, we cannot look further at the multiple physiological characteristics that might be part of genetic differentiation.

The alternative approach is the isolation of strains from surface and deep samples followed by genetic analysis. Its well-known disadvantage is that selection during the process of isolation can result in isolation of strains or species that are not the most abundant ones. Isolation can also be difficult and time-consuming for oligotrophic cyanobacteria because of their slow growth rate (around 1 doubling per day or less at low light levels) (28). Its advantage is that strains in the laboratory can be physiologically characterized, and other ecological techniques become possible. One can use immunofluorescence techniques, for example (3), to look at strain distributions in more detail. We are pursuing both PCR and isolation strategies to look at cyanobacterial speciation in the California Current. We report here on the isolation of strains from two depths from the oligotrophic edge of the California Current, their phylogenetic relationships as seen through *rpoC1* gene sequences, and preliminary characterization of the relative PUB/PEB ratios of some isolates.

MATERIALS AND METHODS

Isolation and growth of strains. Samples were obtained in 1993 at the oligotrophic edge of the California Current at station 83.110 of the CalCOFI (California Cooperative Oceanic Fisheries Investigations, cruise 9304) grid pattern. Surface (5 m) and deep (95 m) samples were incubated under low light during the remainder of the cruise and brought back to the laboratory (approximately 1 week later after sampling because of ship travel time), where they were filtered (1.0-μm pore diameter) and enriched with k/10 trace metals (15), 10 μM PO_4, and 100 μM $NaNO_3$ or 50 μM urea. After visible growth (based on color and turbidity) was evident in the enrichments, clonal isolates were obtained by repeated cycles (at least twice) of colony isolation from pour platings essentially as described by Brahamsha (2). The efficiency of pour plating is 70 to 100% for an open ocean marine *Synechococcus* isolate in laboratory studies (2).

The resulting isolates were clonal and unialgal, but some contained heterotrophic bacterial contaminants, and thus they are not yet axenic. Strains have been maintained in the original media or in SN medium (29) with 1/10 (900 μM) of the usual $NaNO_3$ as a nitrogen source.

DNA isolation. DNA templates for PCR amplification were obtained as crude lysates from frozen cell pellets of cyanobacterial cultures, fresh cell cultures, or genomic minipreps of these sources. For preparation of cell lysates, cyanobacterial cultures were harvested at a cell density of approximately 6.1×10^7 cells/ml. A volume of 200 μl (1.2×10^7 cells) was centrifuged at 13,000 × *g* for 2 min; the pellet was resuspended in a final volume of 30 μl of sterile MilliQ water (Millipore) and boiled at 95°C for 5 min in a thermocycler (PTC-100; MJ Research, Inc.). The cell slurry was centrifuged at 13,000 × *g* for 2 min, and 1.5 μl of the supernatant was used as the template in PCR amplifications (100-μl reaction volume). Frozen cell pellets were treated similarly. For those strains that were not PCR amplifiable by these methods, a genomic miniprep was prepared according to the method described by Golden et al. (12) and modified by Brahamsha (2).

PCR amplification of *rpoC1*. The primers used to amplify a 612-bp fragment of the *rpoC1* gene were the same as those described previously (19, 20). The PCR reagents were obtained from Perkin-Elmer and were used at the manufacturer's recommended concentrations (final $MgCl_2$ concentration, 2 mM [pH 8.3]). The PCR amplification procedure included a hot start step in which all components of the reaction except the polymerase were heated at 92°C for 2 min, at which point the polymerase was added. Typical PCR conditions were 35 cycles of 92°C for 1 min, 55 or 58°C for 1 min, and 72°C for 2 min. Optimal annealing temperatures were 55 and 58°C for California Current low-PUB group (CCLPUB) and California Current high-PUB group (CCHPUB) isolates, respectively. This difference might be due to the apparent higher moles percent G+C content of the CCHPUB strains.

Sequencing. Sequencing was done with the ABI PRISM dye terminator cycle sequencing kit with Amplitaq DNA polymerase, FS, (Applied Biosystems) according to the instructions and the amounts of template and primers recommended by the manufacturer. The sequences were obtained on a Perkin-Elmer Applied Biosystems 373 DNA sequencer.

For sequencing of the amplified products of *rpoC1*, two approaches were used. Some PCR products were sequenced directly. Others were first cloned with the TA cloning system (Invitrogen, San Diego, Calif.) into either pCRII or pCR2.1 plasmid vectors according to the manufacturer's instructions. For sequencing of cloned inserts, vector primers on opposite ends of the insert were used: PCRIIF (5′ ATGCATGCTCGAGCGGCCG 3′) and PCRIIR (5′ GTACCGAGCTCG GATCCAC 3′) for the forward and reverse primers, respectively. To obtain the complete sequence of an insert on complementary strands, internal primers were used as described previously and in some cases with the direct sequencing primers described below.

For direct sequencing from the PCR amplification product, one of two GC-biased internal primers was used: 5′ CATGTWTGGTATCTIAAAGG 3′ (AT biased) or 5′ CACGTSTGGTACCTSAAGGG 3′ (GC biased), depending on the expected GC bias of the fragment. Direct sequencing was used to screen some isolates to avoid cloning and sequencing of identical strains. For direct sequencing, PCR products were purified with QIAquick columns (Qiagen, Chatsworth, Calif.) according to the suggestions of the manufacturer. DNA concentrations of the purified PCR products were determined at the optical density at 260 nm.

Phylogenetic analysis. Alignments of the nucleotide and derived amino acid sequences obtained were done with the program Clustal W (24). Phylogenetic trees were constructed with a Jukes-Cantor distance matrix analysis (DNADIST) and the neighbor-joining method (NEIGHBOR), and bootstrap analyses (100 replicates) were done with SEQBOOT, all from the software Phylip 3.572 (8). Maximum parsimony analyses were done with PAUP 3.1 (23).

Pigment and cell analyses. Cultures of strains 9311 (CCLPUB) and 9317 (CCHPUB) were grown at 21°C under a low photon flux density (6.4×10^{14} quanta s^{-1} cm^{-2}) in 1/10 nitrogen SN medium. In vivo absorption spectra of exponentially growing cells were measured with a dual-beam spectrophotometer (Perkin-Elmer) with an integrating sphere. The absorption spectra were normalized to chlorophyll (674-nm wavelength). Fluorescence characteristics of the strains were analyzed by flow cytometry (Becton Dickinson FACSort). Green fluorescent microbeads (0.9-μm diameter; Duke Scientific) were added to cell suspensions as an internal reference. Relative fluorescence, determined by excitation by a 488-nm-wavelength, 15-mW laser, was measured in three channels: FL1 (wavelengths of 515 to 545 nm), FL2 (wavelengths of 564 to 606 nm), and FL3 (wavelengths of 650 nm and longer). Fluorescence was compensated such that no FL1 fluorescence was measured with WH7805, a strain that lacks PUB. In vivo fluorescence from intact cells collected in channels FL1, FL2, and FL3 is thus qualitatively proportional to that of PUB, PEB, and chlorophyll *a*, respec-

TABLE 1. Characteristics of the isolates used in this study

Strain no.	Color in culture (low light)[a]	A_{495}/A_{545} ratio[b]	Original sample depth (m)	Nitrogen source provided during isolation
9302	Brown	1.33	95	Urea
9303	Brown	ND[c]	95	Urea
9305-3	Brown	1.33	95	Nitrate
9305-2	Brown	1.32	95	Nitrate
9306	Brown	1.32	95	Urea
9307	Brown	1.36	95	Urea
9316	Brown	ND	5	Nitrate
9317	Brown	1.24	5	Nitrate
9318	Brown	ND	5	Urea
9305-1	Pink	1.09	95	Nitrate
9305-4	Pink	1.08	95	Nitrate
9308	Pink	1.05	95	Nitrate
9310	Pink	1.10	95	Nitrate
9311	Pink	1.16	95	Nitrate
9314	Pink	1.01	5	Nitrate
mot2	Brown	1.32	Scripps Pier	Nitrate
WH8103		1.31	—[d]	
WH7805		1.156	—	
WH7803		0.979	—	

[a] Brown, CCHPUB; pink, CCLPUB.
[b] In vivo absorbance in cell suspensions without an integrating sphere.
[c] ND, not determined.
[d] —, information on these strains is provided by Waterbury et al. (28).

tively. In particular, ratios of FL1 to FL2 or plots of FL1 versus FL2 provide qualitative indications of PUB/PEB ratios.

Nucleotide sequence accession number. Sequences for strains CC9311, CC9318, CC9317, and CC9305-3 have been deposited in GenBank under accession no. AF013607 to AF013610, respectively. Accession numbers for sequence data for the other strains used in this study can be found in the article by Palenik and Swift (21).

RESULTS

Isolation of strains. A total of 15 clonal cyanobacterial cultures were obtained: 4 from a surface sample and 11 from a deep sample from a site at the oligotrophic edge of the California Current (Table 1). The isolates have different pigment characteristics in culture when grown under low light, and they were assigned to two groups, CCHPUB and CCLPUB. (In subsequent studies, isolates will be designated simply as CC followed by strain numbers.) CCLPUB strains were obtained with $NaNO_3$ as the sole nitrogen source in the enrichment, whereas CCHPUB strains were enriched with both $NaNO_3$ and urea (Table 1). However, all strains can grow on nitrate or urea as a nitrogen source, at least as nonaxenic cultures. All strains are considered to be of the genus *Synechococcus* based on their phylogenetic relationships defined below.

A high-PUB, motile *Synechococcus* strain similar to those described previously (30) was isolated from the near-shore California Current off Scripps Pier (2a) and characterized genetically with *rpoC1*. It has been included here as another California Current isolate, since relatively few such isolates are available.

Nucleotide sequence data from strains. Fragments of *rpoC1* were readily amplified with crude boiled cell lysates from either fresh cell cultures or frozen pellets as templates. Further DNA purification was required only for 9305-2.

The 612-bp *rpoC1* fragments of nine isolates were cloned and partially sequenced. The nucleotide sequences of some isolates were found to be almost identical in the 270-bp C-terminal region. CCLPUB strains 9311, 9314, and 9305-4 were greater than 98% identical. CCHPUB 9318 and 9305-2 were also greater than 98% identical, as were 9317, 9307, and 9303. Only one strain (CCHPUB 9305-3) lacked other closely related representatives. Because initial sequencing results indicated that many of the isolates contained nearly identical sequences, direct sequencing of the PCR products was used to more efficiently screen subsequent isolates. For this reason, for 9305-1, 9310, 9308, 9306, 9316, and 9302, only the sequence of the 270-bp C terminus was obtained. These strains showed greater than 98% sequence identity to previous lineages. The direct sequencing was thus useful as a screening method. Only one representative of each of four identified lineages was considered for further phylogenetic analyses with all 612-bp (Table 2). Sequence data for both strands were obtained for these clones and for some additional strains.

As done previously (19), the potential codon bias in the third codon position of the four lineages was examined. Over the 612-bp region, the G+C contents of the third codon position were 60, 70, 67, and 74 mol% for CCLPUB9311, CCHPUB 9317, CCHPUB9318, and CCHPUB9305-3, respectively. These results suggest that CCLPUB9311 may have an overall lower moles percent G+C content than the other strains, but we do not know if these differences are significant. Clearly they are not as dramatic as those seen previously between some shotgun-cloned sequences.

TABLE 2. Percent identity matrix for strains presented in Fig. 2[a]

Strain	% Identity											
	9311	9317	9305-3	9318	WH8103	WH7803	WH7805	PCC7942	MED	SS120	PCC6307	ELake
9311		82	82	82	81	82	83	74	70	76	78	80
9317	81		92	89	84	81	82	74	70	74	82	83
9305-3	79	93		89	86	83	84	74	70	74	83	84
9318	80	88	87		82	83	83	74	71	73	82	83
WH8103	76	83	84	81		84	83	75	68	71	85	85
WH7803	82	82	84	84	83		91	74	68	73	85	84
WH7805	81	83	83	84	81	91		75	68	74	82	83
PCC7942	67	68	68	69	68	68	67		65	66	76	76
MED	70	65	66	68	63	65	66	59		77	65	65
SS120	75	71	72	70	70	73	74	59	75		69	70
PCC6307	75	82	82	81	84	84	79	70	61	67		94
ELake	76	81	82	81	82	82	80	68	62	67	94	

[a] The upper right shows the percent identity for the *rpoC1* 612-bp fragment, and the lower left shows data for the 270-bp C terminus. WH8103, WH7803, and WH7805 are the reference strains of *Synechococcus*; MED is a *Prochlorococcus* strain; SS120 is a *Prochlorococcus* strain; PCC6307 is a *Synechococcus* strain; PCC7942 is a *Synechococcus* strain; and ELake is the Elizabeth Lake isolate.

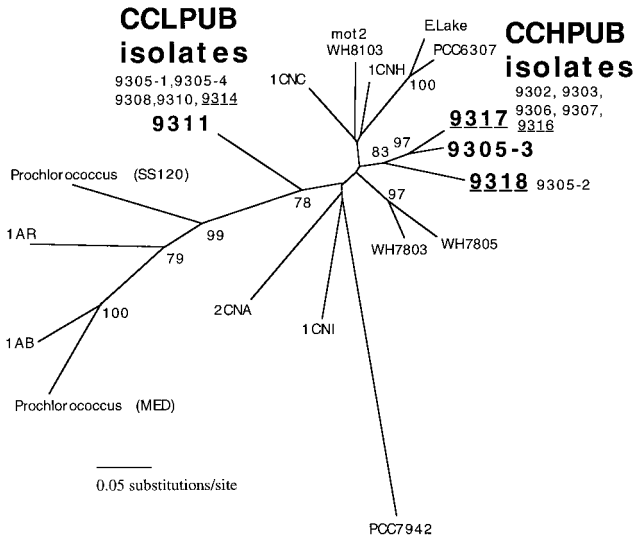

FIG. 1. Phylogenetic tree constructed with Jukes-Cantor-corrected distances and the neighbor-joining method for the analyzed C-terminal 270-bp fragment of *rpoC1* from selected shotgun-cloned sequences from the Sargasso Sea (1CNC, 1CNH, 2CNA, 1CNI, 1AR, and 1AB) and isolated strains. California Current strains isolated from the 5-m-depth sample are underlined, whereas the remaining strains were obtained from a 95-m-depth sample. Smaller numbers indicate strains that bear high similarity (98 to 100%) to the representative strain (boldface type) of each lineage. Bootstrap values greater than 50 are shown for each node. E. Lake, Elizabeth Lake.

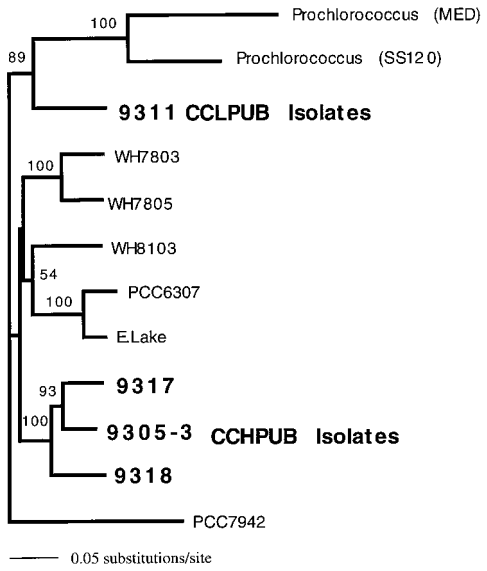

FIG. 2. Phenogram constructed with Jukes-Cantor-corrected distances and the neighbor-joining method for the 612-bp *rpoC1* gene fragment. Designations are as in Fig. 1. Bootstrap values greater than 50 are shown in the corresponding nodes.

Phylogenetic analyses. RNA polymerase *rpoC1* sequence data were analyzed by distance matrix and neighbor-joining methods and by maximum parsimony (data not shown). Using only the C-terminal region, analyses revealed *Synechococcus*-like strains (defined by PCC7942, PCC6307, and the WH isolates) that fell into two highly divergent genetic groups. One group included isolates 9311, 9314, 9305-1, 9305-4, 9310, and 9308, while the other included isolates 9318, 9317, 9316, 9307, 9306, 9305-3, 9305-2, 9303, and 9302. These two major groups corresponded to the two pigment types (CCLPUB and CCH PUB [see below]). There was a higher diversity among CCH PUB strains represented by three less-divergent lineages, 9317 (and 9316, 9307, 9306, 9303, 9302), 9305-3, and 9318 (and 9305-2), whereas CCLPUB had much less genetic diversity within the group (Fig. 1).

The 270-bp C-terminus fragment allowed a comparison between the sequences of our isolates and those obtained as shotgun-cloned sequences from the Sargasso Sea (19). This confirmed that CCLPUB and CCHPUB represented genetic groups not previously detected (Fig. 1). In contrast, the motile strain from the California Current (mot2 from Scripps Pier) exhibited a high degree of similarity (99.67% identity over 612 bp, 100% identity over 270 bp) to motile strains isolated from the North Atlantic (WH8103) (Fig. 1), suggesting that motile strains may form a closely related phylogenetic group.

To better resolve phylogenetic relationships, the 612-bp data set was also analyzed. This analysis yielded the same phylogenetic tree seen for the 270-bp data set (Fig. 2), but showed higher bootstrap values for the branches. One notable finding was that the CCLPUB group (isolate 9311) appears to represent the *Synechococcus* group most closely related to *Prochlorococcus* known to date, and bootstrap values for this branch are highly significant.

Pigment and cell analyses. The in vivo absorption spectra of 9317 (CCHPUB) and 9311 (CCLPUB) show the same peaks due to the presence of PUB and PEB, but at different relative proportions (Fig. 3). The PUB/PEB ratios (A_{495}/A_{545} ratio [28]) measured without an integrating sphere for most of the California Current isolates were about 1.3 and 1.1 for high- and low-PUB strains, respectively, while those of standard culture collection isolates grown under the same conditions were 1.31 and 0.98 for WH8103 and WH7803, respectively. These are similar but not identical to 2.40 and 0.39 for WH8103 and WH7803, respectively, as measured by Waterbury (28).

The relative proportions of pigments in a cell determine the fluorescence excitation and emission characteristics of the cell.

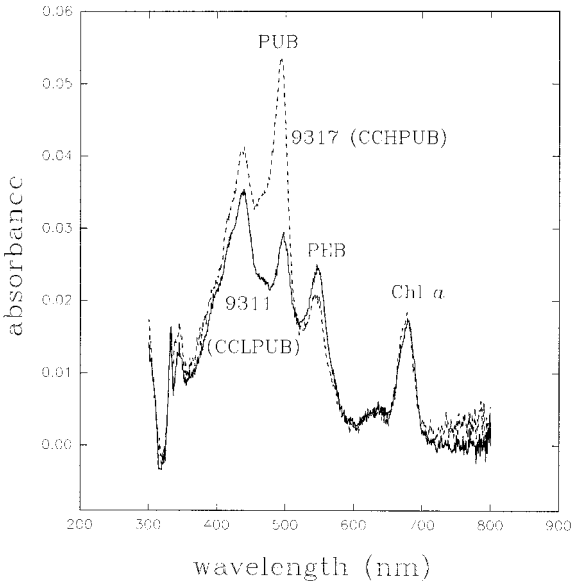

FIG. 3. In vivo absorption spectra of the strains 9311 (CCLPUB) and 9317 (CCHPUB), normalized to chlorophyll *a* (Chl *a* [674 nm]) peak.

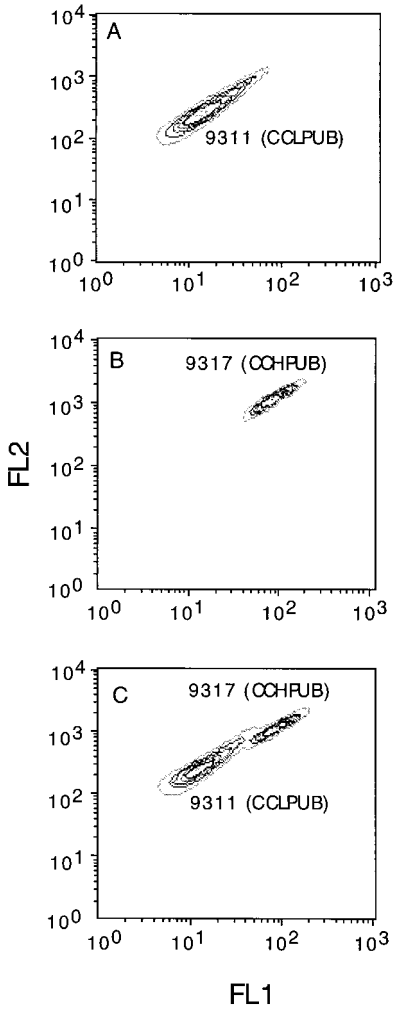

FIG. 4. Flow cytometric signature of CCLPUB and CCHPUB isolates under low light. Fluorescence (relative units) on FL1 is proportional to PUB, whereas that on FL2 is proportional to PEB. The cytogram represents cyanobacterial populations of 9311 (CCLPUB) (A), 9317 (CCHPUB) (B), or both strains in the same laboratory sample (C). Contours correspond to increasing cyanobacterial cell densities, which were gated, and are presented in the logarithmic scale.

When cyanobacterial autofluorescence is excited with a 488-nm argon laser in a flow cytometer, cyanobacteria with different pigment characteristics can be differentiated by the ratios of fluorescence in different wavelength bands (for example, FL1 and FL2 [see Materials and Methods]) (32). When representative CCHPUB and CCLPUB strains were grown under low-light conditions and analyzed by flow cytometry, they were readily identifiable in a mixed laboratory sample with an FL1 versus FL2 contour plot (Fig. 4). When grown under high-light conditions, however, it was much more difficult to distinguish the strains (not shown). These results could be used to interpret the presence of high- and low-PUB population signatures in field samples from the California Current (see Discussion) as being due to the CCHPUB and CCLPUB genetic groups, but this is only highly speculative at this time.

DISCUSSION

As reviewed by Waterbury et al. (28), the group *Synechococcus* encompasses a wide range of freshwater, terrestrial, and marine isolates with different pigments, moles percent G+C content, and other physiological characteristics. Within marine *Synechococcus*-like isolates, efforts have been directed at looking at overall diversity, and the findings suggest likely differences at the genus level (27).

A related goal is to understand the relationships between genetic diversity and environmental conditions, but little work has been done in terms of examining coexisting marine *Synechococcus* strain diversity for a single site or sample. In some cases, isolates exist but have not been genetically characterized (28). Previously, *Synechococcus* sp. strains WH7803 and WH7805 were isolated from the same geographical region on different days, but these appear to be relatively similar (Fig. 1). The presence of high- and low-PUB strains isolated from the same sample cannot be taken itself as evidence of genetic distance, unfortunately. WH7805, although closely related to WH7803, actually lacks PUB altogether.

We show here that at least two (and likely more) genetically distinct and relatively divergent groups of *Synechococcus* strains coexist in the oligotrophic edge of the California Current, as seen with *rpoC1* gene sequence data. These two groups are genetically distinct from model strains currently used in laboratory studies (WH7803, WH7805, and WH8103) and from sequences obtained from a bulk DNA sample from the Sargasso Sea (Fig. 1). Although the latter sample showed the presence of divergent *Synechococcus*-like groups, the data here show for first time with actual isolates, that genetically divergent *Synechococcus* groups coexist in the same seawater sample.

The two phylogenetic groups described in this study clearly show a correlation with PUB/PEB ratios under low-light, nutrient-replete conditions (Table 1 and Fig. 3). Pigment ratios, however, do not uniquely define these groups, because other *Synechococcus* strains (WH7803, for example) have low PUB/PEB ratios as well.

The task of understanding overall *Synechococcus* strain diversity in the California coast environment seems tractable in the sense that only two major groups were found. This diversity is likely to be higher, however, because the genetic diversity of isolates obtained with enrichment culture methods is often thought to be highly biased. In this case, bias may have been introduced because of the necessary lag period of transporting samples back to the laboratory for culturing. It may also have been due to the presence of urea or nitrate as nitrogen sources. Some marine *Synechococcus* strains are known to lack the genetic capability of using urea (4, 28), and other strains might preferentially use ammonium rather than nitrate or urea and thus would have been outcompeted by those more amenable to growth in the enrichments.

The diversity among these isolates in relation to total diversity can be evaluated in the future by comparing their *rpoC1* sequences to those of shotgun-cloned sequences from bulk DNA samples from the same environment. The natural abundances and/or relative distributions of the different groups detected in this study could also be assessed by using sequence data from *rpoC1* to develop group-specific oligonucleotide probes or PCR primers for use in quantitative probing (10, 13) or PCR-based assays. Also, the value of having isolates is that immunofluorescent approaches become possible (3).

In conjunction with the measurement of in situ distributions, future physiological characterization of the strains, including the effects of light and nutrients on pigment ratios, should provide a picture of the ecological niches, if they exist, of the CCHPUB and CCLPUB groups. Unexpectedly, we have not as yet found a clear correlation between the depth of isolation of a strain and its genetic identity. This is in contrast to Olson et al. (17), who found that high-PUB-signature strains, as de-

tected by flow cytometry, were more prevalent in deeper samples and further offshore. These results have also been replicated in the California Current (4a). Again, simply because of water column mixing, in situ competition kinetics, and selection during isolation, one can isolate what are predominantly surface strains at depth and vice versa. However, the fact that we were able to isolate identical or nearly identical strains at the surface and deeper depths suggests that the large gradients with depth of light intensity, light quality, and nutrients are not as high a selective pressure as was hypothesized. More quantitative approaches will thus be required to characterize the distributions of different genetically defined groups.

Diversity within the major groups is also an important issue. There was obviously more diversity within the CCHPUB group than within the CCLPUB group. Are these true differences in clonal diversity between major groups, brought about by processes such as random genetic drift, viral selection, etc., or are these isolation artifacts? We presently have little information by which to judge the significance of these levels of genetic divergence or what they might mean in terms of the divergence of physiological capabilities.

The recent discovery of phycoerythrin in the *Prochlorococcus* Sargasso isolate (14) further supports the phylogenetic relationship between *Synechococcus* and the *Prochlorococcus* group seen with 16S rRNA (25) and *rpoC1* (20, 21). *Synechococcus* sp. strain CCLPUB 9311, being the closest known lineage (at least based on *rpoC1*) to *Prochlorococcus*, will make it a key model organism with which to further study the evolutionary divergence between these two groups. With the advent of genetic manipulation of marine *Synechococcus* strains (2), it may be possible to genetically alter CCLPUB 9311 by knocking out the phycobilisome and adding genes for the synthesis of chlorophyll *b*, essentially making it a prochlorophyte. Although this would be a lengthy process, through this kind of project, we would truly understand the changes necessary for the transition from phycobiliprotein to chlorophyll *b*; a similar, possibly independent, transition may have been involved in the origin of the green chloroplast and higher plant lineages (20, 25).

ACKNOWLEDGMENTS

We thank NSF Biological Oceanography grant OCE96-33111 for funds supporting this research and CONACYT for partial fellowship support (92755) of G.T. NSF funds BIR93-17958 also made possible the purchase and use of the Becton Dickinson FACSORT.

We thank B. G. Mitchell and T. Moisan for help with the integrating sphere spectrophotometer and J. Collier for help running the flow cytometer. We thank M. Ferris, J. Collier, and B. Brahamsha for comments on the manuscript and B. Brahamsha for providing clonal strain mot2 for *rpoC1* analysis.

REFERENCES

1. **Bergsland, K. J., and R. Haselkorn.** 1991. Evolutionary relationships among eubacteria, cyanobacteria, and chloroplasts: evidence from the *rpoC1* gene of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **173:**3446–3455.
2. **Brahamsha, B.** 1996. A genetic manipulation system for oceanic cyanobacteria of the genus *Synechococcus*. Appl. Environ. Microbiol. **62:**1747–1751.

2a. **Brahamsha, B.** Unpublished observations.

3. **Campbell, L., E. J. Carpenter, and V. J. Iacono.** 1983. Identification and enumeration of marine chroococcoid cyanobacteria by immunofluorescence. Appl. Environ. Microbiol. **46:**553–559.
4. **Collier, J. L., and B. Palenik.** 1996. Urea as nitrogen source for marine *Synechococcus*, p. 72. *In* Abstracts of the Meeting of the American Society for Limnology and Oceanography 1996, EOS, Transactions. American Geophysical Union, Washington, D.C.

4a. **Collier, J. L., and B. Palenik.** Unpublished observations.

5. **Douglas, S. E., and N. Carr.** 1988. Examination of genetic relatedness of marine *Synechococcus* spp. by using restriction fragment length polymorphisms. Appl. Environ. Microbiol. **54:**3071–3078.
6. **Eppley, R. W.** 1986. Plankton dynamics of the Southern California Bight. Springer, Berlin, Germany.
7. **Ernst, A., P. Marschall, and C. Postius.** 1995. Genetic diversity among *Synechococcus* spp. (cyanobacteria) isolated from the pelagial of Lake Constance. FEMS Microbiol. Ecol. **17:**197–204.
8. **Felsenstein, J.** 1993. PHYLIP (Phylogeny Inference Package) version 3.5p. Department of Genetics, University of Washington, Seattle. (Distributed by the author.)
9. **Ferris, M. J., G. Muyzer, and D. Ward.** 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. **62:**340–346.
10. **Giovannoni, S., M. S. Rappe, K. L. Vergin, and N. L. Adair.** 1996. 16S rRNA genes reveal stratified open ocean bacterioplankton populations related to the green non-sulfur bacteria. Proc. Natl. Acad. Sci. USA **93:**7979–7984.
11. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature **345:**60–63.
12. **Golden, S. S., J. Brusslan, and R. Haselkorn.** 1987. Genetic engineering of the cyanobacterial chromosome. Methods Enzymol. **153:**215–231.
13. **Gordon, D. A., and S. J. Giovannoni.** 1996. Detection of stratified microbial populations related to *Chlorobium* and *Fibrobacter* species in the Atlantic and Pacific Oceans. Appl. Environ. Microbiol. **62:**1171–1177.
14. **Hess, W. R., F. Partensky, G. van der Staay, J. M. Garcia-Fernandez, T. Borner, and D. Vaulot.** 1996. Coexistence of phycoerythrin and chlorophyll a/b antenna in a marine prokaryote. Proc. Natl. Acad. Sci. USA **93:**11126–11130.
15. **Keller, M. D., R. C. Selvin, W. Claus, and R. R. L. Guillard.** 1987. Media for the culture of oceanic ultraphytoplankton. J. Phycol. **23:**633–638.
16. **Klenk, H. P., and W. Zillig.** 1994. DNA-dependent RNA polymerase subunit B as a tool for phylogenetic reconstructions—branching topology of the Archaeal domain. J. Mol. Evol. **38:**420–432.
17. **Olson, R. J., S. W. Chisholm, E. R. Zettler, and E. V. Armbrust.** 1990. Pigments, size, and distribution of *Synechococcus* in the North Atlantic and Pacific Oceans. Limnol. Oceanogr. **35:**45–58.
18. **Palenik, B.** 1992. Polymerase evolution and organism evolution. Curr. Opin. Genet. Dev. **2:**931–936.
19. **Palenik, B.** 1994. Cyanobacterial community structure as seen from RNA polymerase gene sequence analysis. Appl. Environ. Microbiol. **60:**3212–3219.
20. **Palenik, B., and R. Haselkorn.** 1992. Multiple evolutionary origins of prochlorophytes, the chlorophyll b-containing prokaryotes. Nature **355:**265–267.
21. **Palenik, B., and H. Swift.** 1996. Cyanobacterial evolution and prochlorophyte diversity as seen in DNA-dependent RNA polymerase gene sequences. J. Phycol. **32:**638–646.
22. **Postius, C., A. Ernst, U. Kenter, and P. Boger.** 1996. Persistence and genetic diversity among cyanobacteria from the picoplankton of Lake Constance. J. Plankton Res. **18:**1159–1166.
23. **Swofford, D. L.** 1993. PAUP: phylogenetic analysis using parsimony (version 3.1.1). Illinois Natural History Survey, Champaign, Ill.
24. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
25. **Urbach, E., D. L. Robertson, and S. W. Chisholm.** 1992. Multiple evolutionary origins of prochlorophytes within the cyanobacterial radiation. Nature **355:**267–270.
26. **Venrick, E. L.** 1990. Phytoplankton in an oligotrophic ocean-species structure and interannual variability. Ecology **71:**1547–1563.
27. **Waterbury, J. B., and R. Rippka.** 1989. Subsection I. Order *Chroococcales* Wettstein 1924, emend. Rippka et al., 1979, p. 1728–1729. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. Williams & Wilkins, Baltimore, Md.
28. **Waterbury, J. B., F. W. Watson, F. W. Valois, and D. G. Franks.** 1986. Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus,* p. 71–120. *In* T. Platt and W. K. W. Li (ed.), Photosynthetic picoplankton. Canadian Department of Fisheries and Oceans, Ottawa, Canada.
29. **Waterbury, J. B., and J. M. Willey.** 1988. Isolation and growth of marine planktonic cyanobacteria. Methods Enzymol. **167:**100–105.
30. **Waterbury, J. B., J. M. Willey, D. G. Franks, F. W. Valois, and S. W. Watson.** 1985. A cyanobacterium capable of swimming motility. Science **230:**74–76.
31. **Wilmotte, A.** 1995. Molecular evolution and taxonomy of cyanobacteria, p. 1–25. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
32. **Wood, A. M., P. K. Horan, K. Muirhead, D. A. Phinney, C. M. Yentsch, and J. B. Waterbury.** 1985. Discrimination between types of pigments in marine *Synechococcus* spp. by scanning spectroscopy, epifluorescence microscopy, and flow cytometry. Limnol. Oceanogr. **30:**1303–1315.
33. **Wood, A. M., and D. Townsend.** 1990. DNA polymorphism within the WH7803 serogroup of marine *Synechococcus* spp. (Cyanobacteria). J. Phycol. **26:**576–585.