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Resolution of *Prochlorococcus* and *Synechococcus* Ecotypes by Using 16S-23S Ribosomal DNA Internal Transcribed Spacer Sequences

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Cultured isolates of the marine cyanobacteria *Prochlorococcus* and *Synechococcus* vary widely in their pigment compositions and growth responses to light and nutrients, yet show greater than 96% identity in their 16S ribosomal DNA (rDNA) sequences. In order to better define the genetic variation that accompanies their physiological diversity, sequences for the 16S-23S rDNA internal transcribed spacer (ITS) region were determined in 32 *Prochlorococcus* isolates and 25 *Synechococcus* isolates from around the globe. Each strain examined yielded one ITS sequence that contained two tRNA genes. Dramatic variations in the length and G+C content of the spacer were observed among the strains, particularly among *Prochlorococcus* strains. Secondary-structure models of the ITS were predicted in order to facilitate alignment of the sequences for phylogenetic analyses. The previously observed division of *Prochlorococcus* into two ecotypes (called high and low-B/A after their differences in chlorophyll content) were supported, as was the subdivision of the high-B/A ecotype into four genetically distinct clades. ITS-based phylogenies partitioned marine cluster A *Synechococcus* into six clades, three of which can be associated with a particular phenotype (motility, chromatic adaptation, and lack of phycourobilin). The pattern of sequence divergence within and between clades is suggestive of a mode of evolution driven by adaptive sweeps and implies that each clade represents an ecologically distinct population. Furthermore, many of the clades consist of strains isolated from disparate regions of the world's oceans, implying that they are geographically widely distributed. These results provide further evidence that natural populations of *Prochlorococcus* and *Synechococcus* consist of multiple coexisting ecotypes, genetically closely related but physiologically distinct, which may vary in relative abundance with changing environmental conditions.

In open-ocean ecosystems, carbon fixation is dominated by the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. Together they have been shown to contribute between 32 and 80% of the primary production in oligotrophic oceans (14, 21, 24, 60). *Prochlorococcus* is closely related to the marine cluster A *Synechococcus*, based on analyses using gene sequences from 16S rRNA (16S rDNA) and *rpoC1*, a subunit of DNA-dependent RNA polymerase (37, 59). However, the two genera have very different light-harvesting systems. *Prochlorococcus* contains divinyl chlorophyll *a* (chl a_2) and both monovinyl and divinyl chlorophyll *b* (chl *b*) as its major photosynthetic pigments, rather than chlorophyll *a* and phycobiliproteins that are typical of cyanobacteria (7, 8, 13).

Cultured isolates of *Prochlorococcus* have been divided into two genetically and physiologically distinct groups, referred to as ecotypes because their differing physiologies have implications for their ecological distributions (28, 31, 44). High-B/A isolates have larger ratios of chl b/a_2 and are able to grow at extremely low irradiances (less than 10 μmol of quanta[Q] m^{-2} s^{-1}), where low-B/A isolates are incapable of growth. Low-B/A

isolates have lower chl b/a_2 ratios and are able to grow maximally at higher light intensities, where high-B/A isolates are inhibited (28). The ecotypes also differ in their sensitivity to copper toxicity, with low-B/A isolates able to grow at free cupric ion concentrations five times higher than those high-B/A isolates can tolerate (26). Furthermore, all low-B/A isolates tested to date are incapable of using nitrate or nitrite as a nitrogen source, while some high-B/A isolates can grow on nitrite (30).

The high- and low-B/A ecotypes were originally named for their difference in optimal growth irradiance (low- and high-light adapted, respectively [31]). However, considering that the ecotypes are differently "adapted" to a multitude of environmental factors, the pigment ratio, a property of the cell rather than of the environment in which it thrives, is a better descriptor for the ecotypes (28, 44). Furthermore, the chl b/a_2 ratio, unlike the growth response to a range of irradiances, is a phenotype that can be rapidly and easily measured in a novel isolate.

The 16S rDNA sequences of the *Prochlorococcus* ecotypes correlate with their physiology. Strains of the low-B/A ecotype are phylogenetically very closely related (99% identity in 16S rDNA sequence) and form a clade well supported by bootstrap values (31, 44, 58). Strains of the high-B/A ecotype have a lower degree of identity in their 16S rDNA sequence (97 to 98%) and are not monophyletic but instead form at least three

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independent branches (44). High-B/A *Prochlorococcus* strains also have a higher degree of sequence identity to marine cluster A *Synechococcus* strains than the low-B/A ecotype. In fact, branching orders between some high-B/A *Prochlorococcus* strains and marine cluster A *Synechococcus* strains are not well resolved using 16S rDNA sequences (31, 44, 58).

The strains assigned to marine cluster A *Synechococcus* are genetically and physiologically diverse (64). All contain phycoerythrins as their major light-harvesting pigments, and some possess the chromophore phycourobilin (PUB), which can attach to phycoerythrin in combination with phycoerythrobilin (PEB) (33). The relative amounts of PUB and PEB vary among strains, and some strains are able to chromatically adapt by changing the ratio of their chromophores in response to different wavelengths of light (35). In addition, some isolates are capable of a novel form of swimming motility (6, 65, 66). *Synechococcus* strains also vary in the G+C content of their genomes and in the ability to utilize organic nutrient sources such as urea (10, 65).

Genetic diversity in marine cluster A *Synechococcus* strains has been examined in a few strains using 16S rDNA sequences (58) and more extensively using *rpoC1* sequences (54, 55). Using *rpoC1*, a collection of strains from the California Current could be divided into two lineages consistent with their high or low PUB amounts. However, each of these lineages was distinct from the typical laboratory model high- and low-PUB strains (WH 8103 and WH 7803, respectively), suggesting that pigment content alone may not resolve the multiple ecotypes of marine cluster A *Synechococcus*. Motility, however, does appear to be correlated with phylogeny, as all motile isolates characterized to date are closely related (55). Another recently described clade consists of strains whose members are capable of altering their pigment content in response to light quality in an acclimation process known as chromatic adaptation (35).

In most eubacteria, the genes for rRNA are organized in operons, with the genes encoding the 16S, 23S, and 5S rRNAs separated by internal transcribed spacer (ITS) regions (15). The ITS contains antitermination box B-box A motifs which prevent premature termination of transcription (5) and also have a role in holding the secondary structure of the nascent rRNA for processing to mature rRNAs (1). The spacer between the 16S and 23S rRNA genes can encode 0, 1, or 2 tRNA genes. Because the ITS exhibits a great deal of length and sequence variation, it has been used in many bacterial groups to delineate closely related strains (3, 9, 19). Whole-genome sequences suggest that low-B/A *Prochlorococcus* strains possess a single rRNA operon, while high-B/A *Prochlorococcus* and marine *Synechococcus* strains possess two identical rRNA operons (http://www.jgi.doe.gov/JGI_microbial/html/index.html).

Here we report on use of the ITS as a phylogenetic tool to identify clades which may represent ecologically distinct populations of *Prochlorococcus* and *Synechococcus*. Although isolates in culture collections may not represent the full extent of diversity due to biases introduced by isolation protocols, they provide crucial physiological information to attach to the phylogenetic clusters. By examining a wide range of physiologically diverse isolates of *Prochlorococcus* and marine cluster A *Synechococcus*, we lay the groundwork for informed studies of genetic diversity and distributions in field populations of these oceanic cyanobacteria and provide a framework for interpreting their phenotypic evolution and delineating their taxonomy.

MATERIALS AND METHODS

Isolation of strains. Thirty-two isolates of *Prochlorococcus* from diverse oceanic regions were employed in this study (Table 1). Isolation conditions, physiology, and genetic data have been reported previously for many of the strains (see Table 1). The majority were isolated by filtering seawater through two stacked 0.6-μm-pore-size filters and enriching with nutrients (7). Five (MIT 9302, MIT 9303, MIT 9311, MIT 9312, and MIT 9313) were isolated by sorting on a flow cytometer (31). Seven of the strains (SS120, SS35, SS51, SS2, MED4, SB, and GP2) have been rendered clonal by serial dilution, and one (MED4Ax) has been rendered free of heterotrophic bacteria by plating (45).

Twenty-two of 25 *Synechococcus* strains used in this study (Table 2) are clonal and have been described previously (65). Strain WH 9908 was isolated from Woods Hole in April 1999 (by M. Sullivan), when the water temperature was less than 10°C, and rendered clonal by picking a single colony from an agar plate. Strains C8015, RS9705, and RS9708 were isolated from the Gulf of Aqaba, Red Sea (23). Of the 25 strains, 22 are phycoerythrin-containing marine cluster A strains. Two strains from marine cluster B (WH 8101 and WH 5701) and one freshwater strain assigned to the *Cyanobium* cluster (PCC 6307) were also included (Table 2).

Culture conditions. For physiology experiments, 20-ml batch cultures of *Prochlorococcus* strains were grown in acid-washed 50-ml glass test tubes at 24°C on a 14 h:10 h light-dark cycle under 18 μmol of Q white light m^{-2} s^{-1}. This light level is roughly equivalent to 1% of surface irradiance, which corresponds to a depth of ≈100 m (assuming typical oligotrophic water values for surface irradiance of I_0 = 2,000 μmol of Q m^{-2} s^{-1} and an extinction coefficient of k = 0.045 m^{-1} [18]). Medium was made from 0.2-μm-filtered, autoclaved Sargasso Sea water enriched with Pro2 nutrients (final concentrations: 10 μM NaH_2PO_4, 50 μM NH_4Cl, 100 μM urea, 1.17 μM EDTA, 8 nM Zn, 5 nM Co, 90 nM Mn, 3 nM Mo, 10 nM Se, 10 nM Ni, and 1.17 μM Fe) (28).

For DNA extraction, *Prochlorococcus* strains were grown in 60-ml acid-washed polycarbonate bottles using the medium and culture conditions described above. *Synechococcus* strains were grown for DNA extraction in 100-ml acid-washed flasks in SN medium under constant light (65).

Pigment measurements. The light-dependent physiology of 15 isolates of *Prochlorococcus* was examined by measuring their chl b/a_2 ratios (28). All experiments were performed in triplicate. Cells were acclimated to the experimental conditions (see above) for at least 10 generations before measurements were taken. A known volume (18 to 22 ml) of exponential-phase culture was filtered onto a 25-mm Whatman GF/F under low vacuum, and filters were stored in liquid nitrogen until extraction. Pigments were extracted with 90% acetone according to established protocols (13, 27) and quantified on a spectrophotometer (Becton Dickinson DU640). Unlike high-pressure liquid chromatography, spectrophotometric methods cannot resolve divinyl chlorophyll b_2 from "normal" monovinyl chlorophyll b; thus, total (b_1 + b_2) values are reported. Pigment concentrations were calculated according to the trichromatic equations of Jeffrey and Humphreys (17).

DNA isolation, PCR, and sequencing. DNA was extracted from 50 ml of late-exponential-phase cultures by using a modified protocol involving cetyltrimethylammonium bromide, phenol, and chloroform (2). The ITS/23S fragment was amplified using primers 16S-1247f (CGTACTACAATGCTACGG) and 23S-1608r (CYACCTGTGTCGGTTT). Primer 16S-1247f was designed using available 16S rDNA sequences from *Prochlorococcus* and *Synechococcus* strains (31, 44, 58) and is a perfect match only to cyanobacterial 16S rDNA sequences, as judged by using the Probe Match function of the latest release of the Ribosomal Database Project (25).

Reactions were done in 25-μl volumes with final concentrations of reactants as follows: 0.25 mM deoxynucleoside triphosphates, 0.1 mM each primer, 0.1 to 1 μg of template DNA, and 0.1 to 0.5 U of the high-fidelity polymerase *Pfu* (Stratagene, La Jolla, Calif.). Cycling parameters were 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 6 min, and a final extension at 72°C for 10 min using either a Robocycler (Stratagene) or a PTC100 (MJ Research). Amplified fragments were visualized on agarose gels. Only one band was observed from each culture. Control reactions lacking template DNA were always performed in parallel and gave no products.

For sequencing, PCRs were performed in quintuplicate and pooled, and primers were removed using Strataprep columns (Stratagene). Products were sequenced on an ABI377 or ABI310 (PE Biosystems) automated sequencer using Big Dye terminator sequencing kits according to the manufacturer's instructions. The ITS was sequenced bidirectionally using primer 16S-1247f and primers

TABLE 1. Origins and characteristics of the *Prochlorococcus* strains used in this study[a]

Group and strain	Isolation		Chl b/a_2 ratio	Strain origin[b]	Reference	GenBank accession no.
	Location	Depth (m)				
Low-B/A *Prochlorococcus* clade I						
MED4	Mediterranean Sea	5	0.20	MIT	29	AF397673
MED4Ax[c]	Mediterranean Sea	5	ND	MIT	45	AF397674
MIT 9515	Equatorial Pacific	15	**0.24**	MIT	This work	AF397675
MB11E08	Monterey Bay	0	—		51	AY033307
MB11F02	Monterey Bay	0	—		51	AY033310
Low-B/A *Prochlorococcus* clade II						
AS9601	Arabian Sea	50	**0.34**	R. Olson and A. Shalapyonok	47	AF397677
GP2	West Pacific	150	**0.32**	A. Shimada	48	AF397678
MIT 9107	South Pacific	25	**0.34**	MIT	58	AF397679
MIT 9116	South Pacific	25	**0.31**	MIT	This work	AF397680
MIT 9123	South Pacific	25	**0.34**	MIT	This work	AF397681
MIT 9201	South Pacific	0	0.27	MIT	28	AF397682
MIT 9202	South Pacific	79	0.31	MIT	28	AF397683
MIT 9215	Equatorial Pacific	0	0.30	MIT	28	AF397684
MIT 9301	Sargasso Sea	90	**0.42**	MIT	This work	AF397685
MIT 9302	Sargasso Sea	100	0.51	MIT	31	AF397686
MIT 9311	Gulf Stream	135	**0.47**	MIT	This work	AF397687
MIT 9312	Gulf Stream	135	0.34	MIT	31	AF397688
MIT 9314	Gulf Stream	180	**0.28**	MIT	This work	AF397689
MIT 9321	Equatorial Pacific	50	**0.27**	MIT	This work	AF397690
MIT 9322	Equatorial Pacific	0	**0.28**	MIT	This work	AF397691
MIT 9401	Sargasso Sea	0	**0.34**	MIT	This work	AF397692
SB	West Pacific	40	0.32	A. Shimada	49	AF397693
RS810	Red Sea	80	ND	D. Lindell and A. Post	23	AF397676
High-B/A *Prochlorococcus* clade I						
NATL1A	North Atlantic	30	**0.77**	F. Partensky	40	AF397694
NATL2A	North Atlantic	10	**0.97**	F. Partensky	46	AF397695
PAC1	Tropical Pacific	100	ND	L. Campbell	39	AF397696
High-B/A *Prochlorococcus* clade II						
LG	Sargasso Sea	120	ND	MIT, B. Palenik	7	AF397697
SS2[d]	Sargasso Sea	120	ND	MIT	This work	AF397698
SS35[d]	Sargasso Sea	120	ND	MIT	This work	AF397699
SS51[d]	Sargasso Sea	120	ND	MIT	This work	AF397700
SS120[d]	Sargasso Sea	120	1.41	MIT	7	AF397701
High-B/A *Prochlorococcus* clade III						
MIT 9211	Equatorial Pacific	83	1.37	MIT	28	AF397702
High-B/A *Prochlorococcus* clade IV						
MIT 9303	Sargasso Sea	100	0.97	MIT	31	AF397703
MIT 9313	Gulf Stream	135	0.91	MIT	31	AF397704

[a] GenBank accession numbers for the ITS sequences reported or used here are also shown. Strain information is organized by genetically defined clades described in this work. Chl b/a_2 ratios are listed for *Prochlorococcus* cells grown at 17 to 27 μmol of Q m^{-2} s^{-1}. Values in bold are from this work. Other values are taken from references 28, 29, and 31. —, environmental sequence, no pigment data available. ND, not determined.

[b] MIT, Massachusetts Institute of Technology.

[c] MED4Ax is an axenic derivative of the clonal strain MED4 (45).

[d] Clonal derivative of LG.

internal to the PCR fragment: ITS-Alaf (TWTAGCTCAGTTGGTAGAG), ITS-Alar (CTCTACCAACTGAGCTAWA), and 23S-241r (TTCGCTCGCCRCTACT).

Secondary-structure determination. The complete rRNA operon sequences of *Prochlorococcus* strains MED4 and MIT 9313 and *Synechococcus* strains WH 8102 were obtained from the Department of Energy's Joint Genome Institute (http://www.jgi.doe.gov/JGI_microbial/html/index.html), where the genome sequences of these three strains are near completion. For each of the three strains, ITS sequences obtained from the genome project were identical to those determined independently from PCR products as described above. To simplify the construction of secondary-structure models of the ITS, the 16S rRNA, 23S rRNA, and 5S rRNA sequences were deleted from the predicted transcripts. The remaining sequences were folded using mfold (70). Structures were refined and displayed in LoopDLoop (http://iubio.bio.indiana.edu/soft/molbio/loopdloop/).

Phylogenetic analysis. Sequences were edited and aligned manually based on the predicted secondary structures using the Genetic Data Environment (50) or BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic analyses and calculation of fractional identities and G+C contents used PAUP* version 4b8 (53). Phylogenetic analyses employed either 233 or 434 positions of the 16S-23S rDNA spacer and did not include sequences from the tRNAs.

Distance trees were inferred using minimum evolution as the objective criterion and paralinear (logdet) or HKY85 distances. Distance and maximum-parsimony bootstrap analyses (1,000 resamplings) were performed with heuristic searches utilizing random addition and tree-bisection reconnection branch-swapping methods. Maximum-likelihood analyses used the HKY85 model of nucleotide substitution with rate heterogeneity and empirical nucleotide frequencies. The gamma shape parameter and the transition-transversion ratio were initially estimated from a distance topology and refined by iterative likelihood searches. Likelihood bootstrap analyses (100 resamplings) were performed with heuristic searches and tree-bisection reconnection branch-swapping methods starting from a neighbor-joining tree. Phylogenetic trees were visualized with Treeview (34).

RESULTS AND DISCUSSION

Physiology of *Prochlorococcus* strains. In order to expand the physiological framework for our phylogenetic analyses from that established by Moore and Chisholm (28) based on 10 strains of *Prochlorococcus*, we analyzed the chl b/a_2 ratio of 15

TABLE 2. Origins and characteristics of the *Synechococcus* strains used in this study[a]

Group and strain	Isolation location	A_{495}/A_{545}	Other data[b]	Origin[c]	Reference	GenBank accession no.
Marine A *Synechococcus* clade I						
WH 8015	Woods Hole	0.44		WH	65	AF397717
WH 8016	Woods Hole	0.4		WH	65	AF397718
WH 8020	Sargasso Sea	0.78	CA	WH	65	AF397719
WH 9908	Woods Hole	ND[g]		WH	This work	AF397720
Marine A *Synechococcus* clade II						
WH 6501	Tropical Atlantic	0.43		WH	65	AF397706
WH 8002	Gulf of Mexico	0.48		WH	65	AF397707
WH 8005	Gulf of Mexico	0.44		WH	65	AF397708
WH 8012	Sargasso Sea	0.4		WH	65	AF397709
WH 8109	Sargasso Sea	0.89		WH	65	AF397710
Marine A *Synechococcus* clade III						
C8015	Red Sea	>1	Motile	D. Lindell and A. Post	23	AF397711
WH 8102	Western Caribbean	2.06	Motile	WH	65	AF397712
WH 8103	Sargasso Sea	2.4	Motile, NCA	WH	65	AF397713
WH 8112	Sargasso Sea	Variable	Motile, CA	WH	65	AF397714
WH 8113	Sargasso Sea	Variable	Motile, CA	WH	65	AF397715
WH 8406	Gulf of Mexico	0.84	Motile	WH	65	AF397716
Marine A *Synechococcus* clade IV						
EBAC392	Monterey Bay	—[d]		—	4	AF268237
MB11A04	Monterey Bay	—		—	51	AY033297
MB11E09	Monterey Bay	—		—	51	AY033308
Marine A *Synechococcus* clade V						
RS9705	Red Sea	<1		D. Lindell and A. Post	23	AF397725
RS9708	Red Sea	<1		D. Lindell and A. Post	23	AF397726
WH 7803	Sargasso Sea	0.39		WH	65	AF397727
Marine A *Synechococcus* clade VI						
WH 7805	Sargasso Sea	No PUB		WH	65	AF397721
WH 8008	Gulf of Mexico	No PUB		WH	65	AF397722
WH 8017	Woods Hole	No PUB[e]		WH	65	AF397723
WH 8018	Woods Hole	No PUB		WH	65	AF397724
Marine B *Synechococcus*						
WH 8101	Woods Hole	NA[f]		WH	65	AF397728
WH 5701	Long Island Sound	NA[f]		WH	65	AF397729
Cyanobium Synechococcus						
PCC 6307	Wisconsin lake	NA[f]		PCC	42	AF397730

[a] See Table 1, footnote *a*. A_{495}/A_{545} ratio is a measure of relative amounts of PUB to PEB. Data for Woods Hole collection *Synechococcus* strains are from reference 65. Data for Red Sea *Synechococcus* strains were provided by D. Lindell (personal communication).
[b] CA, chromatic adaptation; NCA, no chromatic adaptation (taken from reference 35).
[c] WH, Woods Hole culture collection; PCC, Pasteur Culture Collection.
[d] Environmental sequence, no pigment data available.
[e] Originally described as containing PUB (65).
[f] Strain lacks phycoerythrin and thus has no PUB or PEB.
[g] ND, not determined.

additional *Prochlorococcus* strains (Table 1) grown at 18 µmol of Q m^{-2} s^{-1} illumination. This growth irradiance was chosen based on previous physiological data (28) because it was a level at which all strains were capable of growth and where ecotypic differences in the chl b/a_2 ratio were pronounced.

At this light level, chl b/a_2 ratios for 13 of the strains fell between 0.2 and 0.5 (Table 1), placing them physiologically in the low-B/A ecotype. The remaining two strains, NATL1A and NATL2A, had much higher chl b/a_2 ratios (0.77 and 0.97, respectively), classifying them as high-B/A strains. In addition, NATL1A and NATL2A exhibited growth and pigment responses over a range of irradiances that were similar to those of the other high-B/A strains (E. Cohen, G. Rocap, and S. W. Chisholm, unpublished data).

With the addition of these pigment data for these 15 strains, it is clear that the majority of the *Prochlorococcus* isolates in culture collections are of the low-B/A ecotype (19 compared to only 6 high-B/A strains among the 25 physiologically charac-

terized strains in this study). This is in spite of concentrated efforts to isolate additional high-B/A strains into culture by sampling from the deep euphotic zone, where they are presumed to predominate. This suggests that there may be an isolation bias for the low-B/A strains. For example, filtration steps used to eliminate *Synechococcus* cells, which are larger, could also preferentially remove larger *Prochlorococcus* cells. Indeed, based on flow cytometric measurements, the high-B/A clade IV strains MIT 9303 and MIT 9313 are the largest *Prochlorococcus* isolates we have in our collection. It is noteworthy in this regard that both of these isolates were obtained by flow cytometric sorting, not filtration. In addition, low-B/A strains have a higher μ_{max} at their optimum growth light intensity than do high-B/A strains (28), which could allow them to take over mixed enrichment cultures if care is not taken to avoid these relatively high light levels.

Sequences of ITS. Sequences of the 16S-23S rDNA ITS region were determined for 32 strains of *Prochlorococcus* and

25 clonal strains of *Synechococcus* (Tables 1 and 2). Although the majority of the *Prochlorococcus* isolates have not been rendered clonal, PCR amplifications yielded single-band products, and few sequence ambiguities were observed. Spacer sequences of all of the strains examined contained genes encoding two tRNAs, for isoleucine and alanine, as has been observed in freshwater *Synechococcus* sp. strain PCC 6803 (56).

Sequencing of the 57 strains resulted in 45 unique ITS sequences. *Prochlorococcus* strains SS120, SS2, SS35, and SS51 (Table 1) are clonal derivatives of the primary culture LG, and all five strains had identical sequences. The axenic strain MED4Ax also had an identical sequence to its parent strain MED4. Strains MIT 9107, MIT 9116, and MIT 9123, coisolates from the same water sample from the South Pacific, were identical to each other, as were coisolates MIT 9312 and MIT 9311 from the Gulf Stream. Strains MIT 9321, MIT 9322, and MIT 9401 also possessed identical ITS sequences, although MIT 9401 was isolated from the Sargasso Sea while the other two are from the Equatorial Pacific. *Synechococcus* strains WH 7805, WH 8008, and WH 8018, isolated from the Sargasso Sea, the Gulf of Mexico, and Woods Hole, respectively (Table 2), also possess identical ITS sequences.

Since the same set of primers was used repeatedly on different templates, it could be argued that the identical sequences are the result of contamination in the PCR. However, many of the identical sequences were prepared several months apart, and other cultures were amplified in the interim that yielded different sequences. Furthermore, in no case did a *Prochlorococcus* culture yield a sequence phylogenetically affiliated with sequences from *Synechococcus* cultures or vice versa, nor did any *Prochlorococcus* culture yield a sequence which was incongruent with its pigment phenotype, as might be expected to have occurred if the identical sequences were the result of chance contamination.

Marked differences in the length of the ITS were observed among the 45 unique sequences (Fig. 1). Lengths of the ITS ranged from 537 bp in *Prochlorococcus* strain MIT 9314 to 1,012 bp in *Synechococcus* sp. strain PCC 6307. Within the *Prochlorococcus* strains, the length differences were strongly correlated with ecotype. All of the low-B/A *Prochlorococcus* strains had ITS regions ranging in length from 537 to 548 bp. The high-B/A *Prochlorococcus* strains had much longer ITS sequences, and there was a larger range of lengths among their sequences (Fig. 1). NATL1A, NATL2A, PAC1, SS120, and MIT 9211 ITS sequences ranged in length from 632 to 693 bp, while MIT 9303 and MIT 9313 had spacers of 831 and 829 bp, respectively. The ITS in marine cluster A *Synechococcus* strains ranged from 747 bp (WH 8017) to 810 bp (WH 8103). The majority of the length difference was in the 3′ end of the spacer (tRNA^{Ala}-23S spacer), which ranged from 255 to 531 bp among the strains examined.

Substantial differences also existed in the G+C content of the ITS (Fig. 1). Whereas the low-B/A *Prochlorococcus* ITS sequences had the lowest G+C content (37 to 39%), the high-B/A *Prochlorococcus* spanned a range of values (37 to 45%). The G+C content of sequences from NATL1A, NATL2A, PAC1, SS120, and MIT 9211 was quite similar to that of the low-B/A isolates, ranging from 37 to 39%. However, MIT 9303 and MIT 9313 had higher G+C contents (44 and 45%, respec-

tively). Values for MIT 9303 and MIT 9313 were in the range of the majority of the marine *Synechococcus* strains (41 to 46% G+C). As with its much longer length, the G+C content of the ITS sequence from *Cyanobium* cluster *Synechococcus* sp. strain PCC 6307 was markedly different (54% G+C) from all of the *Prochlorococcus* and marine *Synechococcus* strains. The majority of the strains had lower G+C contents in the 16S-tRNA^{Ile} spacer and the tRNA^{Ala}-23S spacer than in the more highly conserved tRNAs. In fact, for MED4 the spacer sequence without the tRNA has a G+C content that is the same (30%) as that of the whole genome (http://www.jgi.doe.gov/JGI_microbial/html/index.html).

ITS secondary structure. Complete rRNA operon sequences (derived from the whole genome sequence) from *Prochlorococcus* strains MED4 and MIT 9313 and *Synechococcus* strain WH 8102 were used to predict the secondary structure of the ITS in these three strains (Fig. 2). These proposed structures show conserved features observed in other bacteria (16, 32) and are consistent with general rRNA processing patterns in prokaryotes (1). For example, the spacers on both sides of the 16S rRNA (the 16S upstream spacer and 16S-tRNA^{Ile} spacer) are capable of base pairing with each other (Fig. 2), which may help bring the 3′ and 5′ ends of the 16S rRNA sequence together so it can be cleaved to a mature 16S rRNA (1).

Similarly, the tRNA^{Ala}-23S spacer and the spacer downstream of the 23S rRNA are also capable of base pairing (Fig. 2), which may promote processing of the 23S rRNA. Antitermination motifs (5) can also be identified in these three strains, including the box B, a stem-loop of unconserved sequence which precedes the conserved sequence motif box A. In the region upstream from the 16S rRNA, the box A-like sequence GAUC(C/U)UGGAAAG can base pair with the 16S-tRNA^{Ile} spacer to form a double-stranded processing site. Similarly, in the tRNA^{Ile}-23S spacer, there is a box B spacer loop preceding a box A sequence, GAACCUUGACAA. This box A and the region immediately downstream from it are involved in base pairing with the region downstream of the 23S rRNA to form a double-stranded processing site.

The secondary structures of these three strains and the identification of the double-stranded processing sites allowed the prediction of structures for strains in which sequence upstream of the 16S rDNA and downstream of 23S rDNA was not determined (Fig. 3). The nine strains depicted in Fig. 3 were chosen based on preliminary phylogenetic analyses as being representative of the ITS sequence types in *Prochlorococcus* and *Synechococcus*. As with the three strains described above, a box B loop preceding a box A sequence [GAACCUUGA(C/A)AA] could be identified in all these strains.

Structural details evident in the predicted structures help explain the differences in ITS length between the ecotypes and may be informative characters in their own right. For example, the region immediately upstream of the 23S rRNA forms a cloverleaf in *Synechococcus* sp. strain PCC 6307, which is smaller in the marine *Synechococcus* strains (Fig. 3). This structure is also present in *Prochlorococcus* strain MIT 9313 (Fig. 2B), but is reduced to a single stem-loop in *Prochlorococcus* strain MIT 9211, and this stem-loop has become successively smaller in *Prochlorococcus* strains SS120 and NATL2A and is not present in MED4. The progression of
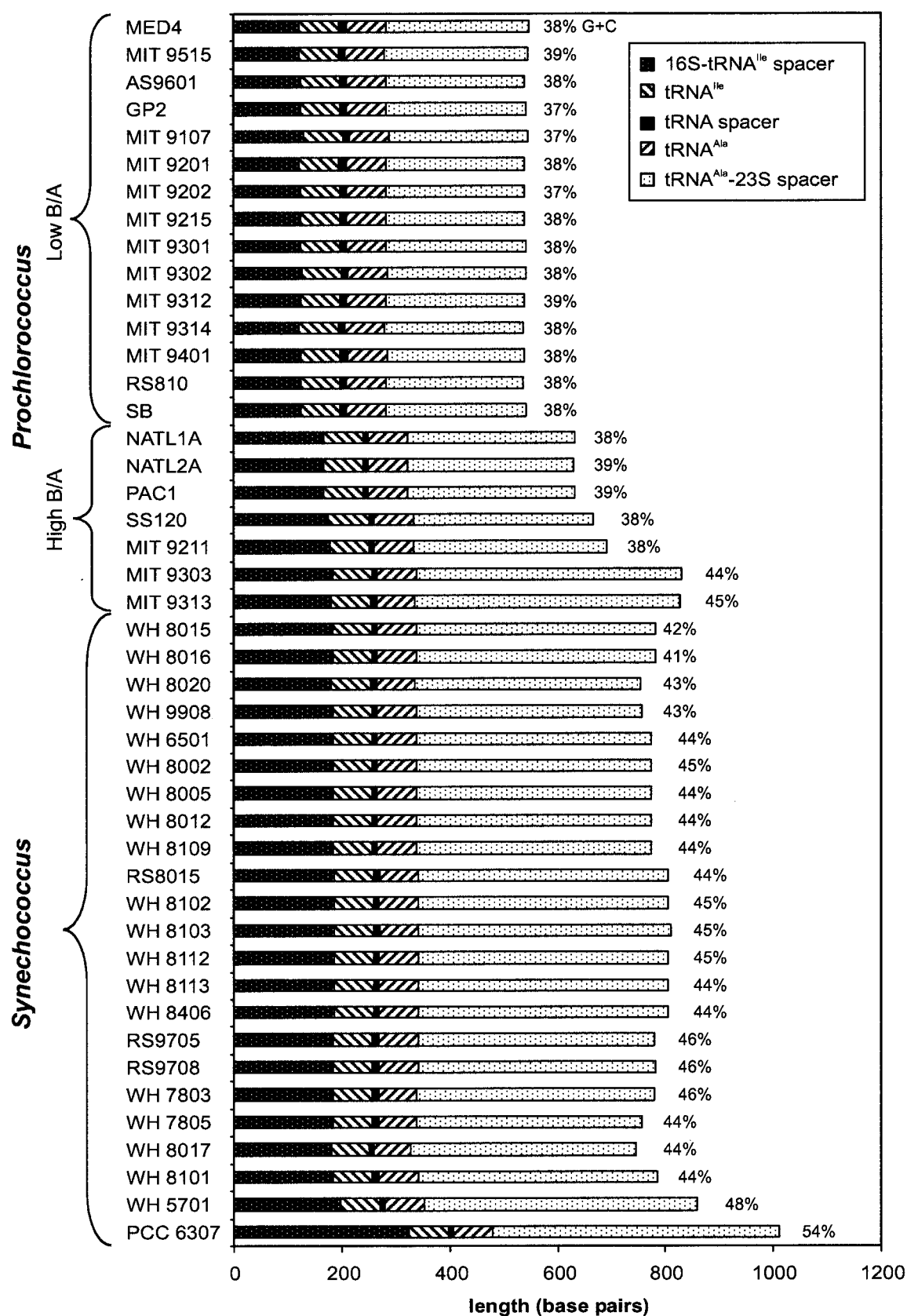
FIG. 1. Length of the ITS region between the 16S and 23S rDNAs in *Prochlorococcus* and *Synechococcus* isolates. Low-B/A *Prochlorococcus* isolates all have short spacers, while high-B/A isolates have longer spacer lengths and are not as similar to one another. The freshwater strain PCC 6307 has a much longer spacer than the marine *Synechococcus* and *Prochlorococcus* isolates. The G+C content of the 16S-23S rDNA ITS is indicated to the right of each bar. Low-B/A *Prochlorococcus* and four high-B/A *Prochlorococcus* isolates have a lower G+C content than two high-B/A *Prochlorococcus* and *Synechococcus* isolates. For sets of isolates with identical ITS sequences (see text), only one isolate is represented here.

A. *Prochlorococcus* MED4

B. *Prochlorococcus* MIT 9313

C. *Synechococcus* WH 8102
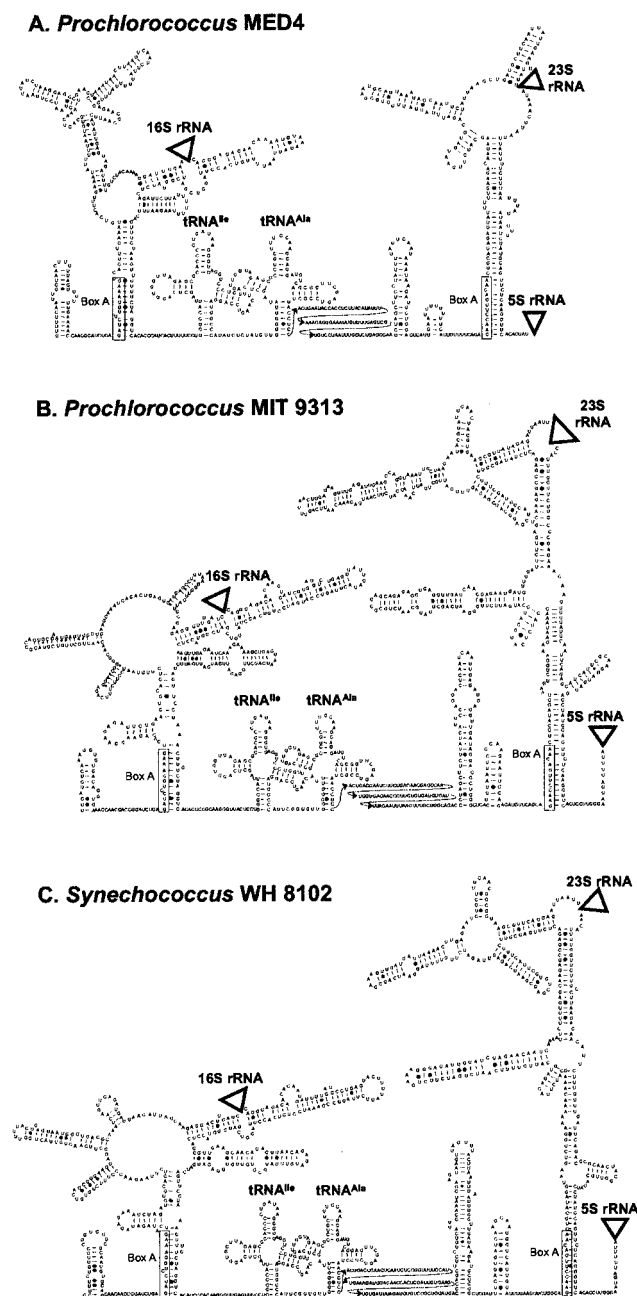
FIG. 2. Predicted secondary structures of the ITS regions of the rRNA operon in (A) *Prochlorococcus* strain MED4, (B) *Prochlorococcus* strain MIT 9313, and (C) *Synechococcus* strain WH 8102. Locations of the 16S rRNA, 23S rRNA, and 5S rRNA are represented by triangles. All strains have antiterminator box B loops and box A sequences conserved in most bacterial ITS sequences, as well as genes for tRNAs isoleucine and alanine typical of cyanobacterial ITS sequences. Sequence corresponding to the box A motif is enclosed in a rectangle. The 5′ region of the tRNA^Ala-23S rRNA spacer (between the tRNA^Ala and the box B loop) for which no structure was inferred is shown in three rows of text to save space.

shorter ITS sequences is consistent with data from the whole-genome sequences of MED4 and MIT 9313, suggesting that there has been an overall genome minimization in MED4 (http://www.jgi.doe.gov/JGI_microbial/html/index.html).

Phylogenetic relationships among *Prochlorococcus* strains. Phylogenetic analyses used alignments based on the inferred secondary structures of the ITS. In phylogenetic analyses confined to 233 bp of the 16S-23S spacer, the low-B/A *Prochlorococcus* strains form a clade (node B) well supported by bootstrap values (Fig. 4A). All of the low-B/A isolates show greater than 93% identity. Within the low-B/A clade there is evidence for at least two subdivisions. One of these, labeled low-B/A II in Fig. 4A, is equivalent to the *Prochlorococcus* low-B/A clade II defined previously by 16S rDNA sequences (44, 67). This clade has high bootstrap support in these analyses, and its members show greater than 95% identity in the ITS sequence.

The remaining low-B/A isolates form a second clade in distance-based analyses but are not monophyletic in the best likelihood tree. On the other hand, likelihood trees in which this group is constrained to be monophyletic are not significantly worse than the best tree, as determined by the Kishino-Hasegawa test. But the monophyly of this clade has low bootstrap support by all methods. Thus, we conclude that there are at least two, and perhaps more, low-B/A clades of *Prochlorococcus*. This is consistent with previous work using 16S rDNA analysis (44, 67), in which two low-B/A clades were resolved.

The high-B/A *Prochlorococcus* strains are not monophyletic, but are dispersed in four distinct lineages (Fig. 4A). One clade, designated high-B/A I, contains isolates NATL1A, NATL2A, and PAC1, which consistently branch together and show a high sequence identity (97%). Isolates SS120 and MIT 9211 have a lower degree of sequence identity to each other (80%) and to the other five high-B/A isolates (less than 83%), and they have each been assigned to a different clade (Fig. 4A). Finally, isolates MIT 9303 and MIT 9313 (99% identity) make up *Prochlorococcus* high-B/A clade IV. This is consistent with the four lineages resolved by 16S rDNA in this ecotype (44).

Given the phylogenetic divergence of the *Prochlorococcus* high-B/A isolates into four clades, the question arises whether the designation high- and low-B/A ecotypes is still adequate for *Prochlorococcus*. Recently, Rippka et al. (41) proposed a subspecies, *pastoris*, to describe the low-B/A *Prochlorococcus* strain PCC 9511, which has a 16S rDNA sequence identical to that of the well-characterized low-B/A isolate MED4. In fact, the high- and low-B/A ecotypes each probably represent a separate species, with each of the clades identified here being a subspecies within the two species. However, until the taxonomy is fully delineated, the designations high- and low-B/A ecotype remain useful. They accurately describe culture physiologies that are correlated with phylogenetic relationships and are convenient terms, to be used with the caveat that the high-B/A ecotype consists of a wider range of physiologies and genetic diversity than the low-B/A ecotype.

The branching order of the low-B/A *Prochlorococcus* clade with respect to the high-B/A clades and *Synechococcus* suggests that the low-B/A clade is more recently arisen, consistent with 16S rDNA analyses (58). If the ability to synthesize divinyl chlorophylls *a* and *b* has been acquired only once (i.e., if the root of the tree is outside the *Prochlorococcus* clade, which is reasonable to assume), then the general possession of divinyl chlorophyll *a* and *b* is a primitive state shared by all *Prochlorococcus* strains and the low-B/A clade is a derived state. This is consistent with a possible evolutionary scenario in which a phycobilisome-containing ancestor acquired the ability to syn-
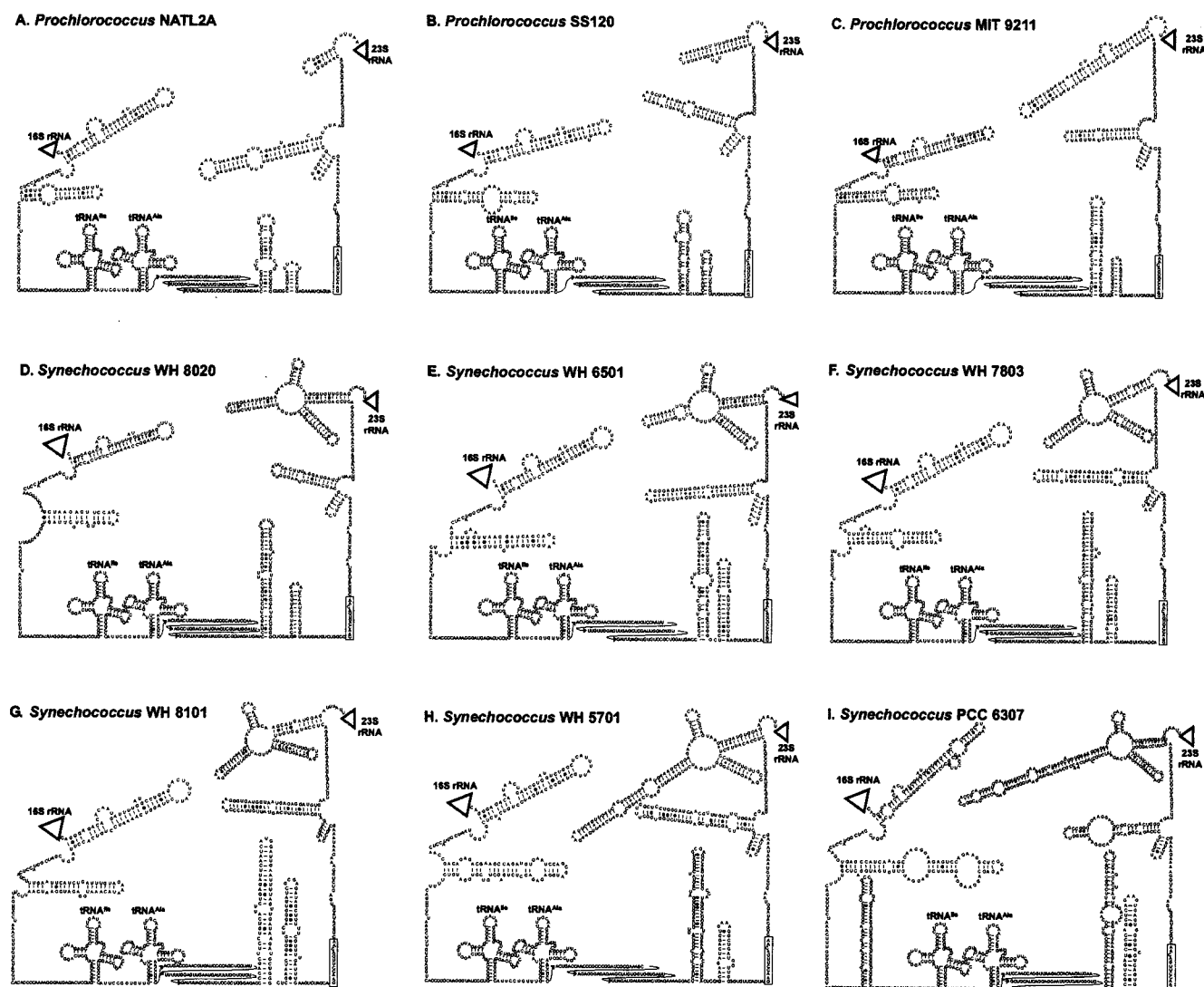
FIG. 3. Predicted secondary structures of the 16S-23S rRNA ITS in three high-B/A *Prochlorococcus* strains and six *Synechococcus* strains identified in this study. (A) *Prochlorococcus* strain NATL2A, (B) *Prochlorococcus* strain SS120, (C) *Prochlorococcus* strain MIT 9211, (D) marine cluster A *Synechococcus* strain WH 6501, (E) marine cluster A *Synechococcus* strain WH 8020, (F) marine cluster A *Synechococcus* strain WH 7803, (G) marine cluster B *Synechococcus* strain WH 8101, (H) marine cluster B *Synechococcus* strain WH 5701, and (I) *Cyanobium* cluster *Synechococcus* sp. strain PCC 6307. Locations of the 16S rRNA, 23S rRNA, and 5S rRNA are represented by triangles. All strains have an antiterminator box B loop and box A sequence conserved in most bacterial ITS sequences as well as genes for tRNAs isoleucine and alanine typical of cyanobacterial ITS sequences. Sequence corresponding to the box A motif is enclosed in a rectangle. The 5′ region of the tRNA[Ala]-23S rRNA spacer (between the tRNA[Ala] and the box B loop) for which no structure was inferred is shown in three rows of text to save space.

thesize divinyl chlorophylls *a* and *b*, allowing it to colonize the deep euphotic zone, where competition for nutrients is reduced, as few other photosynthetic organisms are known to thrive there. Then later modifications, including the ability to survive in higher light and higher copper concentrations characteristic of surface waters, gave rise to the low-B/A clade, which is more competitive throughout the water column.

Phylogenetic relationships among *Synechococcus* strains. Six clades can be identified within the marine cluster A *Synechococcus* strains based on high bootstrap support values and a large degree of within-clade sequence identity (Fig. 4A), which are also well supported in analyses using an expanded subset of 434 positions of the 16S-23S rDNA spacer (Fig. 4B). Three of

these clades are congruent with those identified using *rpoC1*. The first, designated clade I (Fig. 4), contains four strains with 98% identity, one of which (WH 8020) is capable of chromatic adaptation. The second clade (II) consists of low-PUB strains, with 96 to 99% sequence identity. A third well-supported clade (III) consists of motile high-PUB strains that have very high sequence identity (99%).

The remaining three clades cannot yet be compared to those defined by *rpoC1* because there are not enough strains in common. *Synechococcus* clade IV is made up of three environmental sequences that were cloned from Monterey Bay (4, 51). A fifth clade consists of the low-PUB strains WH 7803, RS9705, and RS9708. Finally, clade VI consists of four strains
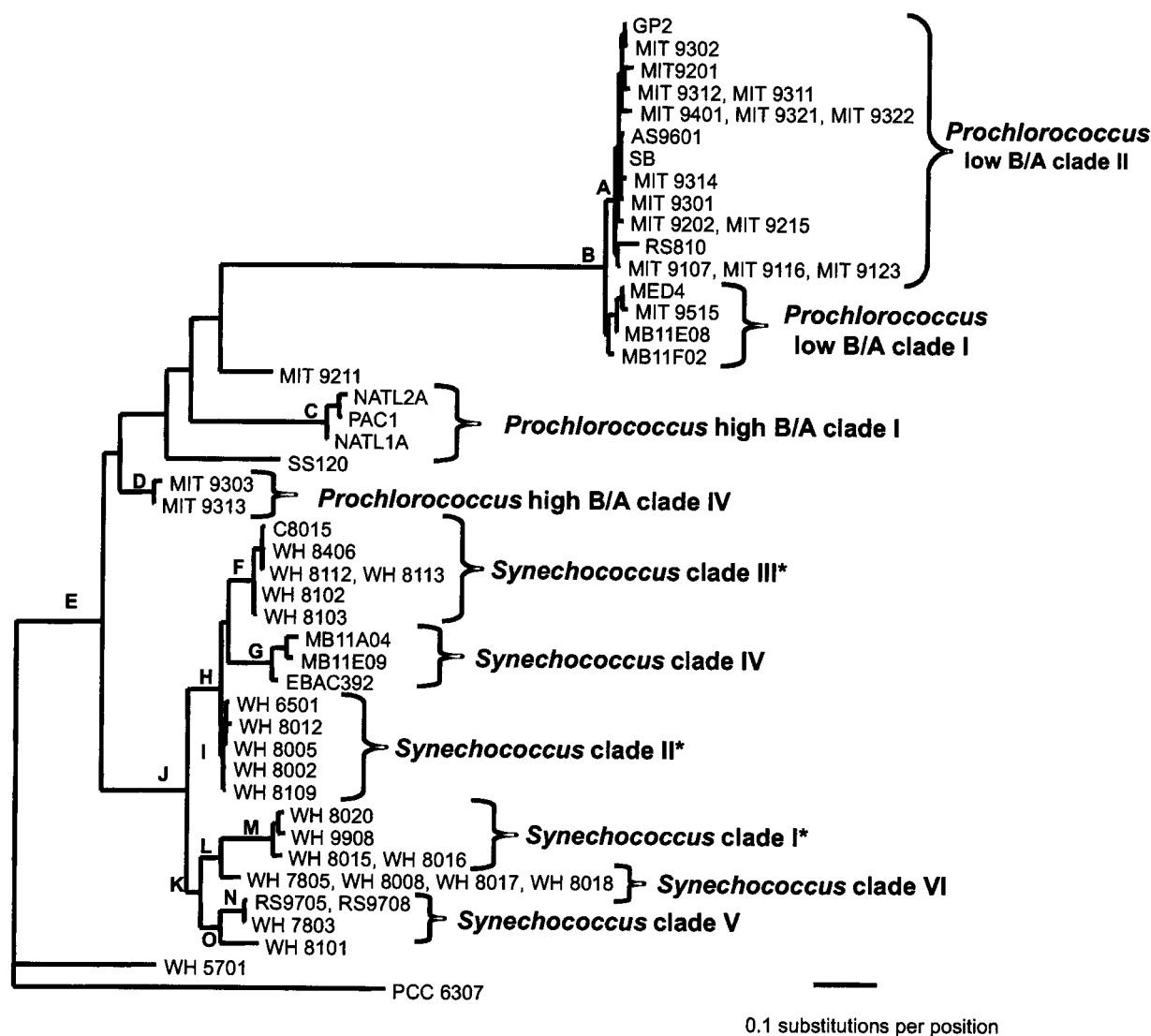
FIG. 4. (A) Evolutionary relationships of *Prochlorococcus* and *Synechococcus* isolates and environmental sequences from Monterey Bay inferred using 233 positions of the 16S-23S rDNA spacer. The tree shown was inferred by maximum likelihood ($-$ln likelihood = 2,364.57) using the HKY85 model of nucleotide substitution with rate heterogeneity (gamma shape parameter = 0.383), empirical nucleotide frequencies, and a transition-transversion ratio of 1.156. The tree shown was not significantly worse (as determined by the Kishino-Hasegawa test) than the best tree found by likelihood ($-$ln likelihood = 2,362.09) in which the *Prochlorococcus* low-B/A clade I was not monophyletic. The *Prochlorococcus* low-B/A clade I was monophyletic in the best trees inferred using HKY85 or paralinear (logdet) distances and minimum evolution as the objective function. The tree is rooted with *Cyanobium* cluster *Synechococcus* sp. strain PCC 6307. Bootstrap values from distance/maximum-parsimony/maximum-likelihood analyses are as follows, with values less than 50 indicated by dashes: A, 79/72/81; B, 100/100/100; C, 100/100/99; D, 100/99/51; E, 64/51/—; F, 100/88/78; G, 100/98/93; H, 97/95/80; I, 100/69/—; J, 90/93/71; K, 55/—/—; L, 60/52/57; M, 100/99/99; N, 99/99/82; and O, 91/87/83. (B) Evolutionary relationships of marine *Synechococcus* isolates inferred using 434 positions of the 16S-23S rDNA spacer. Two strains of high-B/A *Prochlorococcus* and three environmental sequences from Monterey Bay were also included. Asterisks denote clades which are congruent with those identified using *rpoC1*. The phylogenetic framework was determined using paralinear distances (logdet) and minimum evolution as the objective function. Trees inferred using HKY85 distances resulted in identical clades with essentially similar branching orders. Bootstrap values from distance/maximum-parsimony/maximum-likelihood analyses are listed to the left of each node, with values less than 50 indicated by dashes. The tree is rooted with *Cyanobium* cluster *Synechococcus* sp. strain PCC 6307.

which all lack PUB. Three of these, WH 7805, WH 8008, and WH 8018, have identical sequences, while the fourth, WH 8017, is 99% identical. Strain WH 8017 was originally reported to have small amounts of PUB (65), but the reexamination of its pigment content revealed that it lacks PUB and has an absorption spectrum identical to that of WH 8018 (data not shown).

At present it is not possible to discern phenotypes associated with all of the clades of *Synechococcus*. The phycoerythrin composition varies within the clades, suggesting that, with the exception of *Synechococcus* clade IV, whose members all lack PUB, the ratio of PUB to PEB cannot be used as a defining feature within marine cluster A of *Synechococcus*. Without PUB, strains in clade VI are not as efficient in absorbing the
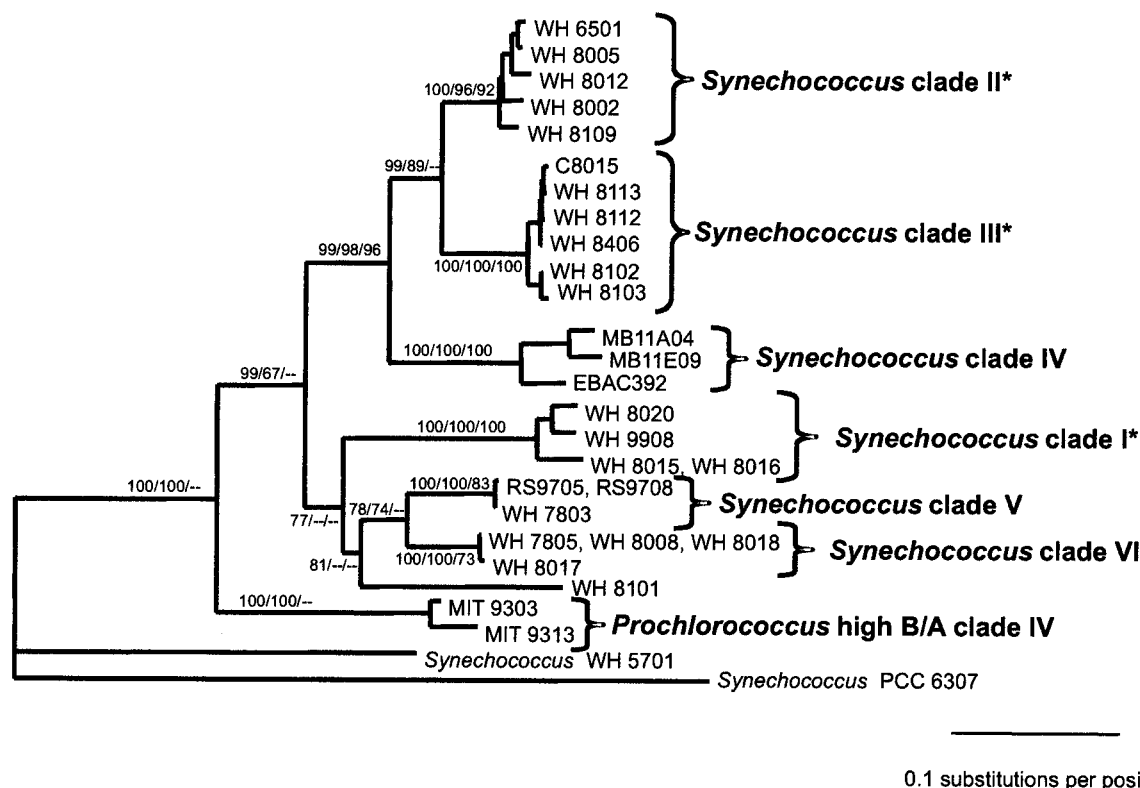
FIG. 4—*Continued.*

blue light characteristic of open ocean waters and may predominate in coastal waters (69).

Two other clades are also associated with characteristic phenotypes. All of the strains in clade III have the ability to swim, consistent with analyses using *rpoC1* in which all motile isolates form a single clade (55). One of the strains in clade I is capable of chromatic adaptation, and this clade may be equivalent to the clade of chromatically adapting strains defined by using *rpoC1* sequences (35). Unfortunately, it is not yet possible to completely compare the clades identified using the ITS with those delineated by *rpoC1* because the majority of the strains used in the two studies are different. Sequencing of both loci in additional strains should enable us in the near future to propose genus and species boundaries within marine cluster A of *Synechococcus*.

Clusters of strains that show greater than 99% identity in their 16S rDNA sequences, such as the low-B/A *Prochlorococcus* strains, are a common feature in the microbial world (62). This may be due to the asexual nature of bacterial reproduction, by which a novel beneficial mutation can sweep through a population, purging it of diversity at most loci while not affecting populations which occupy a different ecological niche and thus are not in direct competition. These adaptive sweeps would decrease diversity within a population and increase diversity between populations. Clusters in which the average sequence divergence between strains of different clusters is more than twice as great as the average sequence divergence between strains of the same cluster may represent ecologically distinct populations (38), and it has been suggested that such

sequence similarity clusters should be at the heart of a natural species concept for bacteria (61). Indeed, in these six clades of *Synechococcus*, the average divergence between clades is always more than twice as great as that within each clade (data not shown), suggesting that the clades do represent ecologically distinct populations, even if we do not at present know how they are all differentiated from one another phenotypically.

Interestingly, the marine cluster B *Synechococcus* strain WH 8101 often branches within the marine cluster A clade V or VI. Although support for these branching orders varies, in no tree topology was WH 8101 more closely related to the marine cluster B strain WH 5701 than to the marine cluster A *Synechococcus* strains. This suggests that the strains now classified as marine cluster B *Synechococcus* may also consist of a number of genetically distinct clades that will require further work to fully characterize.

Global distribution of clades. A striking feature of the clades of both *Prochlorococcus* and marine cluster A *Synechococcus* is their lack of correlation with geographic location of isolation (Tables 1 and 2). This is consistent with results obtained for low-B/A *Prochlorococcus* strains using 16S rDNA sequences (31, 44, 58). At first glance this conclusion is at odds with the detection of low-B/A I but not low-B/A II type sequences in surface waters in the North Atlantic by using probes to whole cells or to amplified 16S rDNA (67, 68). However, the presence of both types of low-B/A sequences in a single water sample has been detected in environmental libraries constructed by using the intergenic region between the photosynthetic elec-

tron transport chain genes *petB* and *petD* and ITS sequences (43, 57). Taken together, these data suggest that all of the clades may be distributed globally but that their relative abundances may change depending on local conditions, as well as seasonally and with depth within a given geographic location.

The examination of sequences directly from the environment, thus avoiding culturing biases, has been widespread in recent years and has given new insights into the diversity of environmental bacteria (12, 63), including marine cyanobacteria (11, 36, 57). In this study one clade of marine cluster A *Synechococcus* (clade IV) is made up entirely of environmental sequences. Thus, even in this well-studied group, culture collections may not yet represent the full extent of genetic and physiological diversity present in natural populations, and additional direct examination of oceanic populations is required.

The ITS is an excellent candidate for direct sequence diversity studies of field populations of marine cyanobacteria because it is variable enough to differentiate the ecotypes unambiguously using restriction fragment length polymorphism (RFLP) or terminal-RFLP analyses. In fact, all six *Prochlorococcus* clades yield distinct RFLP patterns when their amplified ITS sequences are cut with *Hae*II (43). Furthermore, the sequence data presented here allow the design of oligonucleotides specific for each clade that can be used as primers in quantitative PCR (20, 22, 52) to determine the abundances of each clade in the environment.

Concluding remarks. Using the sequences of the 16S-23S rDNA ITS region, we have successfully delineated two important groups of marine cyanobacteria into strain clades which likely represent ecological units. For some of the clades, for example, the low-B/A *Prochlorococcus* strains, some inferences can be made about the nature of the niche that these organisms occupy (high-light surface waters). In other cases, there is not yet an obvious phenotype to explain the different niches these lineages may occupy. The contribution of this work is in identifying the sequence similarity clusters that potentially correspond to ecologically distinct units. This will allow more informed selection of strains in laboratory experiments, as representative strains from each lineage can now be employed in further physiological studies to discover the features of each clade which may have led to niche differentiation. Furthermore, the genetic differences that identify the clades can be used as specific markers to examine their distribution and relative abundances under different environmental conditions, ultimately providing a better understanding of the forces affecting the evolution and population dynamics of this globally successful group of cyanobacteria.

ACKNOWLEDGMENTS

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