Phycobiliprotein genes of the marine photosynthetic prokaryote *Prochlorococcus*: evidence for rapid evolution of genetic heterogeneity

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***Prochlorococcus* is a major photosynthetic prokaryote in nutrient-limited, open ocean environments and an important participant in the global carbon cycle. This phototroph is distinct from other members of the cyanobacterial lineage to which it belongs because it utilizes a chlorophyll *a*₂/*b*₍₂₎ light-harvesting complex as its major antenna, instead of phycobilisomes. Recently, genes encoding the phycobiliprotein phycoerythrin were identified in several *Prochlorococcus* isolates, thus making it the only extant photosynthetic prokaryote to possess a chlorophyll *a/b* antenna as well as phycobiliprotein genes. In order to understand the evolution of phycobiliproteins in this genus, the authors have sequenced the phycoerythrin genes of two isolates that are the most deeply branching in the *Prochlorococcus* lineage and share the highest degree of 16S rDNA sequence similarity to phycobilisome-containing marine *Synechococcus*. Sequence analyses suggest that within the *Prochlorococcus* lineage, the selective forces shaping the evolution of the phycoerythrin gene set have not been uniform. Although strains that are most closely related to marine *Synechococcus* possess genes (*cpeB*, *cpeA*) encoding both subunits of phycoerythrin, a more recently evolved strain is shown to lack *cpeA* and to possess a degenerate form of *cpeB*. Differences in phycoerythrin gene sequences between *Prochlorococcus* and *Synechococcus* appear to be consistent with a model of elevated mutation rates rather than relaxed selection. This suggests that although phycoerythrin is not a major constituent of the light-harvesting apparatus in *Prochlorococcus*, as it is in *Synechococcus*, the *cpeB* and *cpeA* genes are still under selection, albeit a different type of selection than in *Synechococcus*. The evolution of the *Prochlorococcus* light-harvesting antenna complex provides an important system for understanding the origins and scope of phylogenetic diversity in ocean ecosystems.**

Keywords: cyanobacteria, light-harvesting complex, phycoerythrin, phylogeny, relative rates of evolution

INTRODUCTION

The oceans are responsible for nearly half of the earth's photosynthetically fixed carbon. In tropical and subtropical marine environments, the unicellular pro-

Abbreviations: Chl, chlorophyll; PE, phycoerythrin.

The GenBank accession numbers for the sequences reported in this paper are AF304132 (MIT9303 *cpeB*), AF304135 (MIT9303 *cpeA*), AF304133 (MIT9313 *cpeB*) and AF304134 (MIT9313 *cpeA*).

karyotes *Prochlorococcus* and *Synechococcus* are dominant members of the photosynthetic biomass (Partensky *et al.*, 1999). Phylogenetic studies based on analyses of 16S rRNA and other (*psbB*, *psbA*, *petB/D* operon, *rpoC1*) genes indicate that *Prochlorococcus* is most closely related to phycobilisome-containing, marine *Synechococcus* species (marine cluster-A) (Palenik & Haselkorn, 1992; Urbach *et al.*, 1992, 1998; Hess *et al.*, 1995). Branching orders between *Prochlorococcus* and marine *Synechococcus* are not well resolved and suggest

a rapid and almost simultaneous diversification of both groups from a common ancestor (Urbach *et al.*, 1998).

Although *Prochlorococcus* and *Synechococcus* share close phylogenetic ties and several ecological and physiological characteristics, a fundamental difference exists between these genera in their photosynthetic light-harvesting apparatus. A major antenna of the latter consists of phycobiliproteins that are covalently bound to linear tetrapyrrole prosthetic groups and are organized into phycobilisomes (Bryant, 1991). The phycobilisomes of marine cluster-A *Synechococcus* are phycoerythrin (PE)-rich, and two types of PE (I, II) have been identified that differ in their number of associated bilins (Ong & Glazer, 1991; Wilbanks *et al.*, 1991). In contrast, the major antenna complex of *Prochlorococcus* is based on the pigments divinyl chlorophyll *a* (Chl *a*₂) and divinyl and/or monovinyl chlorophyll *b* (Chl *b*₂, Chl *b*) (Chisholm *et al.*, 1992; Goericke & Repeta, 1992; LaRoche *et al.*, 1996; Partensky *et al.*, 1997). Its light-harvesting complex polypeptides (Pcb) are members of the Chl *a*-binding core complex family of antenna proteins, which includes CP43 and CP47 (LaRoche *et al.*, 1996; Partensky *et al.*, 1997).

Recently, genes encoding the α (*cpeA*) and β (*cpeB*) subunits of PE were identified in *Prochlorococcus* sp. strain SS120 (CCMP1375) (Hess *et al.*, 1996). In SS120, both *cpeB* and *cpeA* are found in a gene cluster containing genes that are homologous to those encoding other phycobiliproteins (MpeC, CpeZ) and bilin biosynthesis proteins (Hess *et al.*, 1999). Although low levels of *cpeB* and *cpeA* expression have been detected in SS120 (Hess *et al.*, 1996, 1999), the functional significance of PE in SS120 is unknown. PE genes have since been found in *Prochlorococcus* isolates PAC1 and PAC2 (Penno *et al.*, 2000), and we have also identified *cpeB* in the genome of *Prochlorococcus* MED4. This latter isolate is capable of growing at higher ($> 300 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) irradiance levels than low-light-adapted *Prochlorococcus* isolates such as SS120, and is a member of the high-light-adapted clade (Urbach *et al.*, 1998; Moore *et al.*, 1998; Rocap, 2000).

We have in our collection two low-light-adapted *Prochlorococcus* isolates, MIT9303 and MIT9313, which are the most deeply branching in the *Prochlorococcus* lineage (Moore *et al.*, 1998; Rocap, 2000). In this unique phylogenetic position, their 16S rDNA sequences differ by approximately 2–3% from other isolates of both *Prochlorococcus* sp. and marine *Synechococcus* sp. In order to obtain a more complete understanding of the evolution of phycobiliproteins in *Prochlorococcus*, we sequenced the *cpeB* and *cpeA* genes of both MIT9303 and MIT9313. Our analyses suggest that within the *Prochlorococcus* lineage the selective forces shaping the evolution of the PE gene set have not been uniform. Furthermore, the PE gene sequence heterogeneity we document between *Prochlorococcus* and *Synechococcus* is consistent with a model of elevated mutation rates, rather than relaxed selection. Elucidating the rates and pathways of genetic change in this

important phototroph may help in assessing its response to rapid environmental change.

METHODS

Culture conditions. *Prochlorococcus* isolates MIT9303 and MIT9313, and marine *Synechococcus* sp. strain WH8103, were grown in batch cultures in Sargasso Sea water supplemented with 'Pro2' medium nutrients and trace metals (Moore & Chisholm, 1999). Cultures were maintained at $21 \pm 1^\circ\text{C}$. Illumination was supplied during a 14 h light: 10 h dark cycle by 'cool white' fluorescent lights at $10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Genomic DNA isolation. Genomic DNA was prepared from *Prochlorococcus* according to standard methods (Ausubel *et al.*, 1995) that were optimized for this bacterium (Rocap, 2000). Briefly, cells were lysed in the presence of lysozyme, and DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), precipitated with 2-propanol, and resolubilized in TE buffer (pH 7.6).

PCR amplification, cloning and sequencing. Genes encoding the β - (*cpeB*) and α - (*cpeA*) subunits of PE were amplified from *Prochlorococcus* genomic DNA by PCR. The primers, *cpeB*1.2 (ATGCTTGATGCATTCTCAAG) and *cpeA* 2.2 (AAGGC-ATTAATAAGGTAATC), were designed as exact matches to the SS120 *cpeB* 5'-end and *cpeA* 3'-end (Hess *et al.*, 1996). PCR samples contained 1–3 μg genomic DNA, 0.5 μM of each primer, 250 μM each dNTP, 0.3–5 mM MgCl_2 , cloned *Pfu* buffer ($1 \times$) (Stratagene), and 2.5 units *Pfu* DNA polymerase (Stratagene). Cycle parameters were 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. Samples were processed for 30 or 40 cycles prior to a final extension at 72°C for 10 min.

PCR products were separated on 1.5% agarose gels and visualized by UV fluorescence after staining with ethidium bromide. Selected PCR products of ~ 1000 bp were purified from agarose gels using the QIAEXII Kit (QIAGEN), and then ligated to the pCR-Script Amp SK(+) Cloning Vector (PCR-Script Amp Cloning Kit, Stratagene). Vectors containing this insert were used to transform *Escherichia coli* (Epicurian Coli XL 10-Gold Kan ultracompetent cells, Stratagene). Size-selected clones were sequenced and final *cpeB* and *cpeA* sequences were obtained from three clones for MIT9303 and two clones for MIT9313. Plasmids were sequenced using M13 forward and reverse primers in our laboratory using Sequi-Therm LongRead Cycle Sequencing Kits on LI-COR model 4000L sequencers or by the MITCCR HHMI Biopolymers Laboratory.

Phylogenetic analysis. Sequences used in comparisons were obtained from GenBank and from the complete MED4 genome (http://spider.jgi-psf.org/JGL_microbial/html/). All analyses utilized *cpeB* sequences minus the first 20 nucleotides (5' primer region) and *cpeA* minus the last 24 nucleotides (3' primer region). Data and alignments for 16S rDNA analyses were downloaded directly from the Ribosomal Database Project (RDP) (Maidak *et al.*, 2000).

PE protein sequences were aligned using the CLUSTAL W program with the BLOSUM matrix (Thompson *et al.*, 1994). Gaps were included to optimize the alignments. Only unambiguously aligned positions were used in the phylogenetic analyses (146 amino acids for α -PE, 159 amino acids for β -PE). The percentage identities summarized in Table 1 were calculated from uncorrected distances. Total G+C base content ratios were calculated using GeneMark (Borodovsky

& McIninch, 1993). MEGA version 2.0 (Kumar *et al.*, 2001) was used to calculate the number of synonymous and non-synonymous substitutions using both the original and modified Nei–Gojobori method ($R = 1.28$ for *cpeB*, $R = 1.35$ for *cpeA*) (Nei & Gojobori, 1986; Ina, 1995; Nei & Kumar, 2000; Kumar *et al.*, 2001). The numbers of synonymous and nonsynonymous differences were compared using *p*-distances, where the number of synonymous differences was normalized to the number of synonymous sites, and the number of nonsynonymous differences was normalized to the number of nonsynonymous sites. Conclusions drawn from the original and modified Nei–Gojobori analyses were the same, and only the results of the latter analyses have been reported. Non-parametric relative rate tests were conducted in MEGA version 2.0 (Kumar *et al.*, 2001) using Tajima's general method (Tajima, 1993), with one degree of freedom and a significance level of 5%.

Protein phylogenetic analyses were done with PUZZLE 4.0 (Strimmer & von Haeseler, 1996) for maximum-likelihood, PHYLIP 3.5c (Felsenstein, 1989) for distance, and PHYLIP 3.5c (Felsenstein, 1989) for parsimony algorithms. Distances were calculated in PROTDIST using the Dayhoff–PAM substitution matrix and trees were inferred using the Fitch–Margoliash weighted least-squares criterion in FITCH. Distance and parsimony bootstrap analyses were performed with 1000 pseudo-sampled datasets generated by SEQBOOT. In the protein maximum-likelihood analyses, the JTT substitution model was used and the puzzling step was repeated 1000 times.

PAUP* version 4.0 beta 2a (Swofford, 1999) was used for all nucleotide analyses. Percentage identities reported in Table 6 were calculated from uncorrected distances. Distance trees were inferred using minimum evolution as the objective criterion and paralinear (logdet) distances, which are relatively insensitive to differences in G+C content (Lake, 1994). Nucleotide maximum-likelihood analyses were done via the HKY85 model of nucleotide substitution with rate heterogeneity and empirical nucleotide frequencies. Bootstrap analyses (100 resamplings for maximum-likelihood, 1000 for distance and maximum-parsimony) were performed with heuristic searches utilizing random addition and tree-bisection reconnection branch-swapping methods. Phylogenetic trees were displayed using TREEVIEW (Page, 1996).

Accession numbers. Sequences reported in this paper were deposited in GenBank under the following accession numbers: AF304132 (MIT9303 *cpeB*), AF304135 (MIT9303 *cpeA*), AF304133 (MIT9313 *cpeB*), AF304134 (MIT9313 *cpeA*). Database accession numbers of the PE sequences reported for other organisms, including *Prochlorococcus* (*Pro*) and *Synechococcus* (*Syn*), are as follows: *Pro* SS120 Z68890; *Pro* PAC1 AJ272069; *Pro* PAC2 AJ237612, AJ272030; *Syn* WH7803 X72961; *Syn* WH8103 M91809; *Syn* WH8020 M95288; *Synechocystis* PCC6701 M33812; *Calothrix* PCC7601 X04592; *Pseudanabaena* PCC7409 X63073, S96581; *Porphyra purpurea* U38804; *Aglaothamnion neglectum* Z11907; *Cryptomonas* sp. AFMDB; *Chroomonas* sp. S21775. Accession numbers for 16S rDNA sequences are as follows: *Pro* MIT9303 AF053397; *Pro* MIT9313 AF053399; *Pro* NATL2A AF001467; *Pro* PAC1 AF001471; *Pro* MED4 AF001466; *Pro* SS120 X63140; *Syn* WH8101 AF001480; *Syn* WH8103 AF001479; *Syn* WH7805 AF001478; *Syn* WH7803 AF081834; *Syn* PCC6307 AF001477; *Syn* PCC7001 AB015058; *Syn* PCC6301 X03538; *Syn* PCC7942 D88288; *Synechocystis* PCC6803 D64000; *Microcystis holsatica* U40336; *Microcystis elabens* U40335; *Arthrospira* PCC8005 X70769; *Calothrix* D253 X99213; *Pseudanabaena biceps* PCC7367 AF091108; *Plectonema* F3 AF091110; *Nostoc punctiforme* PCC73102

AF027655; *Pleurocapsa* PCC7516 X78681; *Cyanidium caldarium* chloroplast X52985; *Antithamnion* chloroplast X54299; *Chondrus crispus* chloroplast Z29521; *Palmaria palmata* Z18289; *Cryptomonas* phi chloroplast S73904; *Cyanophora paradoxa* cyanelle U30821.

RESULTS

Amplification, cloning and sequencing of MIT9303 and MIT9313 PE genes

Using primers that were exact matches to the SS120 *cpeB* (5'-end) and *cpeA* (3'-end) genes, and *Synechococcus* sp. strain WH8103 as the control, we optimized the PCR conditions in the presence of sequence mismatches between the primers and the target *cpeB* and *cpeA* genes. Although the PE genes of *Synechococcus* sp. strain WH8103 possessed three mismatches to the *cpeB* primer (5'-end) and six mismatches to the *cpeA* primer (3'-end), we amplified a single DNA fragment of the approximate size expected for *cpeB* and *cpeA* (~1000 bp) (Fig. 1, lane 2). Using the same PCR conditions, we then sought to amplify the genes encoding the α - and β -subunits of PE from the genomic DNA of MIT9303 and MIT9313. Separation of the MIT9303 PCR products on an agarose gel revealed a single major DNA fragment of ~1000 bp (Fig. 1, lane 3). Although this was also the case for MIT9313, additional minor DNA fragments were observed for this isolate (Fig. 1, lane 4). Subsequent cloning and sequencing of the ~1000 bp DNA fragments from the two isolates identified them as putative *cpeA* and *cpeB* genes. Using these gene and derived amino acid sequences, as well as those from SS120 (Hess *et al.*, 1996), we performed a BLAST search of the MED4 genome and were able to identify the MED4 *cpeB* gene. However, we found that MED4 lacks a *cpeA* homologue.

Nucleotide sequence comparisons

The *cpeB* gene sequences of MIT9303 and MIT9313 exhibited 97% identity and were more similar to each other than they were to other *Prochlorococcus* or marine

Fig. 1. PCR amplification of *Prochlorococcus* and marine *Synechococcus* DNA using a set of exact match primers to the 5'-end of SS120 *cpeB* and to the 3'-end of SS120 *cpeA*. The first lane shows a DNA ladder. Lane 1, SS120 (control). Note the single PCR product of ~1000 bp. Lane 2, *Synechococcus* sp. strain WH8103. Lane 3, MIT9303. Lane 4, MIT9313.

Table 1. Identity matrix for *cpeB* (bold type) and β -PE (above the diagonal) and *cpeA* (bold type) and α -PE (below the diagonal) of *Prochlorococcus*, and other cyanophytes and rhodophytes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 <i>Prochlorococcus</i> MIT9303		97	65	65	64	38	67	60	64	61	43	65	61	51	45
2 <i>Prochlorococcus</i> MIT9313	97		65	65	64	38	67	60	64	61	42	52	61	52	46
3 <i>Prochlorococcus</i> SS120	66	67		77	78	40	46	46	59	46	49	39	44	62	65
4 <i>Prochlorococcus</i> PAC1	62	62	70		89	42	49	47	49	48	46	41	44	52	52
5 <i>Prochlorococcus</i> PAC2	61	62	70	94		42	47	45	47	47	47	41	31	52	54
6 <i>Prochlorococcus</i> MED4	—	—	—	—	—		23	16	23	23	30	36	33	37	35
7 <i>Synechococcus</i> WH7803 (PEI)	65	65	59	52	58	—		69	79	68	63	61	63	64	50
8 <i>Synechococcus</i> WH8103 (PEII)	58	57	13	40	40	—	66		55	79	63	61	63	64	58
9 <i>Synechococcus</i> WH8020 (PEI)	69	68	61	53	53	—	79	63		67	66	65	54	66	63
10 <i>Synechococcus</i> WH8020 (PEII)	60	61	42	43	43	—	61	83	63		48	61	54	62	58
11 <i>Synechocystis</i> PCC6701	63	64	61	57	56	—	66	59	63	63		73	69	68	54
12 <i>Calothrix</i> PCC7601	64	63	60	61	53	—	65	59	64	62	80		74	68	55
13 <i>Pseudanabaena</i> PCC7409	61	61	57	58	57	—	71	61	68	60	91	94		59	58
14 <i>Porphyra</i> <i>purpurea</i>	60	60	60	58	52	—	61	54	61	56	65	66	63		78
15 <i>Aglaothamnion</i> <i>neglectum</i>	65	64	32	60	60	—	61	54	65	55	64	65	64	78	

Synechococcus cpeB genes (Table 1). Most notably, of the 14 base-pair changes between MIT9303 and MIT9313, all but one of these changes occurred in the third codon position. Comparable patterns were observed for the MIT9303 and MIT9313 *cpeA* genes (Table 1). Pairwise *cpeB* sequence comparisons among the low-light-adapted isolates (MIT9303, MIT9313, SS120, PAC1; Table 1) indicated that sequence identities ranged from 65% (MIT9303/MIT9313, PAC1) to 97% (MIT9303, MIT9313). However, *cpeB* of MED4 (high-light-adapted clade) exhibited 42% or less nucleotide identity with *cpeB* of other *Prochlorococcus* isolates (Table 1).

The number of synonymous (K_s) and nonsynonymous (K_a) differences for *cpeB* and *cpeA* of *Prochlorococcus* as well as other cyanobacteria, and the K_a/K_s ratios, are reported in Tables 2 and 3, respectively. For both *cpeB* and *cpeA*, K_a/K_s values were not elevated within the *Prochlorococcus* genus relative to marine *Synechococcus* (Table 3). Comparisons between the different genera, as well as between specific strains, indicated that

the range of K_a/K_s ratios was comparable (Table 3). Interestingly, while K_a/K_s was consistently greater than one for *cpeB*, K_a/K_s was less than one for *cpeA*, suggesting that the functional constraints on the evolution of these genes are different.

G + C base ratios

Both the total and third codon G + C base content of *cpeB* and *cpeA* were higher in MIT9303 and MIT9313 than in other *Prochlorococcus* isolates (Table 4). Among the six isolates that have been characterized thus far, the total G + C contents of *cpeB* and *cpeA* in MIT9303 and MIT9313 were closest to those of marine *Synechococcus* sp. For these two genes, the codon usage of MIT9303 and MIT9313 was shifted towards T at the third base position ($T > C > G$ or A). This nucleotide frequency bias was similar to that of other *Prochlorococcus* isolates, whose *cpeB* and *cpeA* genes have third base positions favouring T or A ($T > A > G \geq C$). Similar nucleotide frequency biases towards T ($T > A > C$ or G) have been reported previously for *psbB* and *petB/D*

Table 2. Matrix of the number of synonymous (K_s) and nonsynonymous (K_a) differences for the *cpeB* (above the diagonal) and *cpeA* (below the diagonal) genes of different cyanobacterial genera

Synonymous differences reported here have been normalized using the number of potential synonymous sites. Nonsynonymous differences have been normalized using the number of potential nonsynonymous sites. All values have been multiplied by 100. The upper numbers (bold) indicate the number of synonymous substitutions; the lower numbers indicate the number of nonsynonymous substitutions.

	1	2	3	4	5	6	7	8	9
1 <i>Pro</i> MIT9303		20.4 ± 4.1 35.5 ± 3.1	18.5 ± 4.1 35.2 ± 3.1	38.3 ± 5.1 54.0 ± 3.2	24.2 ± 4.3 33.4 ± 3.1	34.9 ± 4.8 36.1 ± 3.1	26.0 ± 4.4 36.4 ± 3.1	30.8 ± 4.7 40.0 ± 3.2	24.4 ± 4.4 41.9 ± 3.2
2 <i>Pro</i> SS120	40.2 ± 4.7 31.4 ± 2.8		11.4 ± 3.4 26.6 ± 2.8	29.8 ± 4.9 45.8 ± 3.2	32.0 ± 4.8 46.0 ± 3.2	34.7 ± 4.9 47.3 ± 3.2	29.1 ± 4.7 43.6 ± 3.2	29.2 ± 4.7 47.7 ± 3.2	30.9 ± 4.8 39.9 ± 3.1
3 <i>Pro</i> PAC1	50.6 ± 4.8 32.4 ± 2.8	43.1 ± 4.9 24.9 ± 2.6		35.5 ± 5.2 46.5 ± 3.1	26.0 ± 4.5 43.2 ± 3.2	29.7 ± 4.8 47.8 ± 3.2	25.0 ± 4.5 43.8 ± 3.2	22.5 ± 4.4 45.2 ± 3.2	30.0 ± 4.9 42.2 ± 3.1
4 <i>Pro</i> MED4	— —	— —	— —		36.4 ± 5.0 58.1 ± 3.2	42.7 ± 5.2 59.7 ± 3.1	40.1 ± 5.2 56.0 ± 3.2	45.5 ± 5.2 58.6 ± 3.1	37.2 ± 5.2 50.9 ± 3.2
5 <i>Syn</i> WH7803 (PEI)	47.0 ± 4.7 33.5 ± 2.8	56.3 ± 4.7 40.0 ± 2.9	55.1 ± 4.7 38.3 ± 2.9	— —		17.4 ± 3.8 33.4 ± 3.1	12.4 ± 3.3 21.0 ± 2.6	19.7 ± 4.0 36.6 ± 3.1	17.6 ± 3.9 36.0 ± 3.1
6 <i>Syn</i> WH8103 (PEII)	52.5 ± 4.7 37.8 ± 2.9	62.7 ± 4.6 44.3 ± 3.0	64.1 ± 4.6 45.2 ± 3.0	— —	38.6 ± 4.5 31.8 ± 2.8		25.0 ± 4.4 34.2 ± 3.1	13.4 ± 3.4 23.1 ± 2.7	26.5 ± 4.5 44.0 ± 3.2
7 <i>Syn</i> WH8020 (PEI)	37.3 ± 4.6 31.3 ± 2.8	50.2 ± 4.8 38.1 ± 2.9	53.9 ± 4.8 36.3 ± 2.9	— —	34.9 ± 4.4 14.1 ± 2.1	46.9 ± 4.6 34.2 ± 2.9		20.2 ± 4.1 34.1 ± 3.1	20.7 ± 4.2 33.7 ± 3.0
8 <i>Syn</i> WH8020 (PEII)	49.9 ± 4.7 42.1 ± 3.0	55.4 ± 4.8 42.6 ± 2.9	59.6 ± 4.7 43.4 ± 3.0	— —	47.7 ± 4.6 31.7 ± 2.8	30.1 ± 4.3 12.2 ± 2.0	46.5 ± 4.7 36.2 ± 2.9		22.3 ± 4.3 41.0 ± 3.2
9 <i>Syn</i> PCC6701	50.1 ± 4.7 34.1 ± 2.8	51.4 ± 4.8 39.9 ± 2.9	52.2 ± 4.8 37.0 ± 2.9	— —	46.6 ± 4.6 27.4 ± 2.7	54.8 ± 4.6 32.3 ± 2.8	44.4 ± 4.6 30.5 ± 2.8	44.4 ± 4.7 31.7 ± 2.8	

Table 3. Ratios of the number of nonsynonymous to synonymous differences (K_a/K_s) in the *cpeB* (above the diagonal) and *cpeA* (below the diagonal) sequences of different cyanobacteria

	1	2	3	4	5	6	7	8	9
1 <i>Prochlorococcus</i> MIT9303		1.74	1.90	1.41	1.38	1.03	1.4	1.3	1.72
2 <i>Prochlorococcus</i> SS120	0.78		2.33	1.54	1.44	1.36	1.50	1.63	1.29
3 <i>Prochlorococcus</i> PAC1	0.64	0.58		1.31	1.66	1.61	1.75	2.01	1.41
4 <i>Prochlorococcus</i> MED4	—	—	—		1.60	1.40	1.40	1.29	1.37
5 <i>Synechococcus</i> WH7803 (PEI)	0.71	0.71	0.70	—		1.92	1.69	1.86	2.05
6 <i>Synechococcus</i> WH8103 (PEII)	0.72	0.71	0.71	—	0.82		1.37	1.72	1.66
7 <i>Synechococcus</i> WH8020 (PEI)	0.84	0.76	0.67	—	0.40	0.73		1.69	1.63
8 <i>Synechococcus</i> WH8020 (PEII)	0.84	0.77	0.73	—	0.66	0.41	0.78		1.84
9 <i>Synechocystis</i> PCC6701	0.68	0.78	0.71	—	0.59	0.59	0.69	0.71	

Table 4. G + C base ratios of *cpeB* and *cpeA* of *Prochlorococcus* (Pro) and marine *Synechococcus* (Syn)

Strain	Mol% G + C <i>cpeB</i>		Mol% G + C <i>cpeA</i>	
	Total	3rd codon	Total	3rd codon
Pro MIT9303	53	49	49	43
Pro MIT9313	53	49	49	42
Pro SS120	37	17	40	30
Pro PAC1	43	28	38	24
Pro PAC2	41	20	37	23
Pro MED4	28	17	—	—
Syn WH7803 (PEI)	61	72	60	66
Syn WH8103 (PEII)	60	69	60	70
Syn WH8020 (PEI)	54	50	54	49
Syn WH8020 (PEII)	57	57	54	52

genes of several *Prochlorococcus* isolates, including SS120 and NATL2A (Urbach *et al.*, 1998; Partensky *et al.*, 1999), and mutational biases may be a cause of these observed patterns.

Derived β -PE and α -PE amino acid sequences

As indicated in Table 1, *Prochlorococcus* β -PE sequences (excluding MED4) were consistently more similar to each other (77–99% identity) than they were to the β -PEs of marine *Synechococcus* strains (60–67% identity), or other cyanophytes and rhodophytes. Pairwise comparisons between MIT9303/MIT9313 and SS120 revealed that this was most likely because the majority of differences were in the third codon position and many were synonymous. The derived β -PE sequence of MED4 exhibited the lowest amount ($\leq 41\%$) of sequence identity with the β -PE sequences of the other isolates (Table 1). Similar patterns were observed for the α -PE sequences of *Prochlorococcus* (Table 1).

Both the derived β -PE sequences and α -PE sequences exhibited 99% amino acid identity between MIT9303 and MIT9313 (Table 1). While the single amino acid residue difference between β -PE of MIT9303 and MIT9313 involved the conserved substitution of Asp-144 (MIT9303) and Asn-144 (MIT9313), the single residue difference between α -PE involved the semi-conserved substitution of Ala-29 (MIT9303) and Val-29 (MIT9313).

Table 5. Nonparametric relative rate test scores for the β -PE and α -PE sequences of *Prochlorococcus* and other cyanobacteria

A	B	C (outgroup)	χ^2 †	ρ †
<i>Prochlorococcus</i> MIT9303	<i>Prochlorococcus</i> SS120	<i>Synechococcus</i> WH7803(PEI)	1.38 1.96	0.239 0.162
<i>Prochlorococcus</i> MIT9303	<i>Prochlorococcus</i> SS120	<i>Synechocystis</i> PCC6701	1.64 0.62	0.201 0.433
<i>Prochlorococcus</i> MED4	<i>Prochlorococcus</i> MIT9303	<i>Synechococcus</i> WH7803(PEI)	40.50 —	0.00* —
<i>Prochlorococcus</i> MED4	<i>Prochlorococcus</i> MIT9303	<i>Synechocystis</i> PCC6701	37.70 —	0.00* —
<i>Prochlorococcus</i> MED4	<i>Prochlorococcus</i> SS120	<i>Synechococcus</i> WH7803(PEI)	35.06 —	0.00* —
<i>Prochlorococcus</i> MED4	<i>Prochlorococcus</i> SS120	<i>Synechocystis</i> PCC6701	33.38 —	0.00* —
<i>Prochlorococcus</i> MIT9303	<i>Synechococcus</i> WH8020 (PEI)	<i>Calothrix</i> PCC7601	9.26 5.76	0.002* 0.016*
<i>Prochlorococcus</i> MIT9303	<i>Synechococcus</i> WH8020 (PEII)	<i>Calothrix</i> PCC7601	0.00 0.69	1.00 0.405
<i>Prochlorococcus</i> SS120	<i>Synechococcus</i> WH8020 (PEI)	<i>Calothrix</i> PCC7601	20.45 8.80	0.00* 0.003*
<i>Prochlorococcus</i> SS120	<i>Synechococcus</i> WH8020 (PEII)	<i>Calothrix</i> PCC7601	3.60 2.37	0.058 0.123
<i>Prochlorococcus</i> MED4	<i>Synechococcus</i> WH8020 (PEI)	<i>Calothrix</i> PCC7601	56.82 —	0.00* —
<i>Prochlorococcus</i> MED4	<i>Synechococcus</i> WH8020 (PEII)	<i>Calothrix</i> PCC7601	38.63 —	0.00* —
<i>Synechococcus</i> WH8020 (PEI)	<i>Synechococcus</i> WH8020 (PEII)	<i>Calothrix</i> PCC7601	8.10 2.47	0.004* 0.116

† The upper numbers (bold) represent χ^2 or ρ for β -PE analyses; the lower numbers represent χ^2 or ρ for α -PE analyses. Low p values (< 0.05) are marked with an asterisk.

Fig. 2. Alignment of deduced amino acid sequences of β -PE (a) and α -PE (b) of *Prochlorococcus* isolates MIT9303, MIT9313, SS120 and MED4 (β -PE only), marine *Synechococcus* sp. strains WH7803, WH8103 and WH8020, and *Calothrix* sp. strain PCC7601 (formerly *Fremyella diplosiphon*). Identical residues are presented in white type on a black background. Black type on grey squares indicates highly conserved substitutions (within the groups STA NEQK NHQK NDEQ QHRK MILV MILF HY FYW). Putative chromophore-binding sites are indicated with an asterisk above the corresponding Cys residue.

Within the *Prochlorococcus* lineage, relative rate tests for β - and α -PE revealed that mutations in these sequences appear to be accumulating at approximately the same rate for low-light-adapted strains such as MIT9303 and SS120 (χ^2 was not significant at the 5% level) (Table 5). However, the rate at which the β -PE sequence is evolving in MED4 is not the same as that in MIT9303 or SS120. Instead, relative rate tests suggested an elevated rate of evolution for the high-light-adapted MED4 sequence (Table 5).

The results of the relative rate tests between *Prochlorococcus* and *Synechococcus* PE sequences depended on

whether *Synechococcus* PE (I) or (II) was used in the actual test (Table 5). Differences in the rate at which mutations have accumulated in the PE sequences of *Prochlorococcus* strains MIT9303/SS120 and the PE (I) sequence of *Synechococcus* WH8020 were significant (Table 5). However, relative rate tests suggested that mutations have accumulated at approximately the same rate in the *Prochlorococcus* strain MIT9303/SS120 PE sequences as in the *Synechococcus* PE (II) sequences. In the case of MED4, all relative rate tests with *Synechococcus* suggested that the rate at which β -PE is evolving is not equivalent, and that mutations have accumulated faster in MED4 (Table 5).

Fig. 3. Phylogenetic analysis of PE proteins from *Prochlorococcus*, other members of the cyanobacterial lineage, red algae and cryptomonads, using maximum-likelihood, distance and parsimony methods. Likelihood trees are shown for both β -PE (a) and α -PE (b); identical tree topologies were obtained in distance and parsimony analyses. Support values for internal branches (percentage support from quartet puzzling for likelihood or percentage bootstrap values from 1000 resamplings for distance and parsimony analyses) are displayed at each node in the following order: likelihood/distance/parsimony.

Identification of putative functional residues

Alignments of the derived amino acid sequences of the β -PE subunit of MIT9303 and MIT9313 indicated that many residues known for their functional importance in the β -PE subunits of other organisms (Apt *et al.*, 1995) were conserved in all of the *Prochlorococcus* sequences,

excluding MED4 (Fig. 2a, b). These included residues that serve as putative chromophore attachment sites (Cys-50, Cys-61, Cys-82, Cys-162), function in chromophore interaction and stabilization (Arg-77, Arg-78, Ala-80/81, Arg-84, Asp-85), and have a role in subunit interactions (Asp-13, Arg-91, Tyr-92, Tyr-95, Arg-108) (Apt *et al.*, 1995). In contrast, the MED4 β -PE sequence

Fig. 4. Phylogenetic relationships among *Prochlorococcus* and other organisms based on 16S rDNA sequences. The distance tree is shown with bootstrap values for all three methods displayed in the following order: likelihood/minimum evolution-distance/parsimony.

lacked several of these highly conserved residues, including Cys-50(43), Cys-162(155), Ala-80(73)/81(74) and Tyr-92(85) (Fig. 2a).

Alignment of the derived α -PE sequences indicated that several residues important in chromophore attachment or protein interactions were also conserved in this subunit (Fig. 2b). Most notably, both MIT9303 and MIT9313 possessed a cysteine residue at position 130 that is highly conserved among other cyanobacteria and red algae and functions in chromophore binding (Apt *et al.*, 1995). However, this Cys-130 residue is not conserved in the α -PE sequences of any other *Prochlorococcus* isolate (Fig. 2b). In SS120, this position contains an aspartic acid residue (Hess *et al.*, 1996), which does not represent a conserved substitution.

Phylogenetic analyses

Phylogenetic trees constructed using protein sequences deduced from *cpeB* and *cpeA* genes (Fig. 3a, b) revealed that the position of the *Prochlorococcus* cluster relative

to marine *Synechococcus* was not congruent with 16S rDNA based phylogenetic groupings (Fig. 4). *Prochlorococcus* β -PE and α -PE sequences did not group with either type I or type II β -PE and α -PE sequences from marine *Synechococcus* (Fig. 3a, b). However, within the *Prochlorococcus* limb, the clustering of isolates based on both β - and α -PE sequences was consistent with their positions in the 16S rDNA-based tree. As in ribosomal trees, MIT9303 and MIT9313 are very closely related (Table 6, Fig. 4) and form the basal branch of the *Prochlorococcus* clade. Similar tree topologies and levels of support were obtained in trees rooted using phycocyanin sequences and in trees constructed using *cpeB* and *cpeA* nucleotide sequences (both all positions and only the first two codon positions) (data not shown). The MED4 β -PE sequence was not included in the tree (Fig. 3a) because it is highly degenerate and shared low sequence identity ($\leq 36\%$) with other cyanobacterial and red algal β -PEs (Table 1). This resulted in its branch length being five times longer than any of the others in the tree. In phylogenetic trees, the placement of such

Table 6. 16S rDNA sequence identity matrix for *Prochlorococcus* (Pro) and *Synechococcus* (Syn)

	2	3	4	5	6	7	8	9
1 <i>Pro</i> MIT9303	98.97	97.47	97.28	97.19	97.00	98.41	98.23	97.75
2 <i>Pro</i> MIT9313		97.94	98.13	98.03	97.66	97.75	97.19	97.28
3 <i>Pro</i> SS120			98.32	98.03	98.32	97.57	97.00	97.29
4 <i>Pro</i> PAC1				99.53	98.78	97.19	96.72	96.91
5 <i>Pro</i> NATL2A					98.50	96.91	96.44	96.82
6 <i>Pro</i> MED4						97.00	96.72	96.91
7 <i>Syn</i> WH7803							98.60	98.13
8 <i>Syn</i> WH8103								98.41
9 <i>Syn</i> WH8101								

long branches is often at odds with true evolutionary position (Swofford *et al.*, 1996).

DISCUSSION

16S rDNA-based phylogenetic analyses suggest that *Prochlorococcus* and marine *Synechococcus* share a common, phycobiliprotein-containing ancestor, and may have both diversified at a similar point in time (Urbach *et al.*, 1998). Although *Prochlorococcus* has evolved to use a chlorophyll $a_2/b_{(2)}$ light-harvesting complex as its major antenna, it still retains genes encoding the phycobiliprotein PE (Hess *et al.*, 1996; LaRoche *et al.*, 1996; Ting *et al.*, 1999). The branching order we observed among *Prochlorococcus* PE genes (not including the degenerate MED4 *cpeB*) was congruent with previous 16S rDNA sequence analyses for this genus. Furthermore, both complete sequence and third codon G + C base frequencies of *cpeB* and *cpeA* were correlated with 16S rDNA phylogenetic tree branching patterns, with isolates such as MIT9303 and MIT9313 exhibiting the highest (0.48–0.53) G + C base content ratios for these particular genes. A similar correlation between third codon position G + C base frequencies and the branching patterns of 16S rRNA gene phylogenetic trees was demonstrated previously for several *Prochlorococcus* and *Synechococcus* genes (Urbach *et al.*, 1998; Roca, 2000). In particular, other G + C-rich genes identified in MIT9303 include *psbB* (0.52), *petB* (0.58) and *petD* (0.58) (Urbach *et al.*, 1998). Although these data support the idea that the PE genes of *Prochlorococcus* were unlikely to have been acquired by lateral gene transfer (Hess *et al.*, 1999), they do not completely rule out the possibility of recent horizontal transfer from a close relative, particularly in the case of MED4. Nevertheless, no MED4-like *cpeB* sequence has been previously described in any other photosynthetic organism. Thus it is most parsimonious to presume that the PE genes were retained from the phycobilisome-containing ancestor of *Prochlorococcus* and *Synechococcus*.

Our sequence analyses suggest that within the *Prochlorococcus* lineage, the selective forces shaping the evolution

of the PE gene set have not been uniform. Notably, this is apparent in the unique loss of the gene encoding the α -PE subunit in the MED4 strain compared to more deeply branching isolates such as MIT9303 and MIT9313. Loss of PE genes has been suggested to have occurred in other cyanobacterial lines (Apt *et al.*, 1995). MIT9303 and MIT9313 have the highest degree of 16S rDNA sequence similarity to marine *Synechococcus* relative to other cultured *Prochlorococcus* isolates (Table 6), and only they have retained a highly conserved putative chromophore binding site (Cys-130) in their α -PE subunit. Furthermore, relative rate tests suggest that mutations have not accumulated at an equivalent rate in the β -PE sequences of *Prochlorococcus*. In MED4, the presence of a degenerate form of β -PE, characterized by several point mutations and deletions at highly conserved sites, is consistent with an elevated rate of evolution. In addition, the ratios of nonsynonymous (K_a) to synonymous (K_s) substitutions were not elevated in MED4 relative to other *Prochlorococcus* or *Synechococcus* strains. Thus, even though MED4 lacks the gene encoding the α -PE subunit, these results suggest that its *cpeB* gene is still under selection, although likely a different type of selection than in other *Prochlorococcus* strains. As a high-light-adapted strain of *Prochlorococcus*, MED4 is thought to have arisen more recently than its low-light-adapted counterparts (e.g. MIT9303, MIT9313, SS120, PAC1) (Urbach *et al.*, 1998; Roca, 2000), and thus could have been subjected to different selective pressures (i.e. light and/or nutrient availability), leading to dissimilar genetic recombination and/or deletion events.

Although *cpeB* and/or *cpeA* expression has been detected in SS120 (Hess *et al.*, 1996, 1999), and in MIT9303 and MIT9313 (C. Ting, unpublished results), the exact role of PE in the *Prochlorococcus* genus remains to be established. PE is clearly not the major constituent of the light-harvesting apparatus in any extant *Prochlorococcus* strain (Chisholm *et al.*, 1992; Goericke & Repeta, 1992; LaRoche *et al.*, 1996; Partensky *et al.*, 1997), as it is in *Synechococcus* (Ong & Glazer, 1991; Wilbanks *et al.*, 1991). In SS120, the PE genes are part of a larger cluster, within which other phycobiliprotein-related genes (*cpeZ*, *cpeY*, *mpeX*,

ppeC) can be found (Hess *et al.*, 1999). But there is no evidence that PE forms part of a cyanobacterial-like phycobilisome structure in *Prochlorococcus* (Chisholm *et al.*, 1988; Fields *et al.*, 1997; C. S. Ting and others, unpublished). It remains to be explored whether the phycobiliproteins of *Prochlorococcus* are located within the intracytoplasmic lamellar space, as in cryptophytes (Gantt *et al.*, 1971).

Our phylogenetic analyses based on PE sequences show that the position of the *Prochlorococcus* cluster relative to *Synechococcus* was not congruent with 16S rDNA-based trees. Additional analyses indicated that the ratios of nonsynonymous (K_a) to synonymous (K_s) substitutions for the *cpeB* and *cpeA* genes were not elevated in *Prochlorococcus* relative to *Synechococcus*. Instead, K_a/K_s ratios were similar between these genera. This suggests that the sequence differences we observed may be due to elevated mutation rates rather than relaxed selection. This is consistent with the results of relative rate tests between *Prochlorococcus* β/α -PE and *Synechococcus* β/α -PE (I) sequences. Thus although PE has a different function in *Prochlorococcus*, these results suggest that the *cpeB* and *cpeA* genes are still under selection, albeit a different type of selection than in *Synechococcus*.

Future work on the role of PE in *Prochlorococcus* may help to identify whether this protein confers a biological advantage under specific environmental conditions. However, with the change in selection on the PE genes we have observed within the *Prochlorococcus* lineage, it is unlikely that the function of PE will be conserved among all strains. Comparisons between *Prochlorococcus* and *Synechococcus* provide a striking example of how the evolution of a key protein complex, the light-harvesting antenna system, has proceeded along very different paths in two globally important marine prokaryotes.

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