Plant Molecular Biology **32:** 1103–1115, 1996.
© 1996 *Kluwer Academic Publishers. Printed in Belgium.*

Regulation, unique gene organization, and unusual primary structure of carbon fixation genes from a marine phycoerythrin-containing cyanobacterium

Gregory M.F. Watson and F. Robert Tabita*
*Department of Microbiology and the Plant Molecular Biology/Biotechnology Program, The Ohio State University, 484 West 12th Ave, Columbus, OH 43210-1292, USA (*author for correspondence)*

Received 28 June 1996; accepted in revised form 18 September 1996

Key words: carboxysomes, evolution, ribulose 1,5-bisphosphate carboxylase/oxygenase, *Synechococcus*

Abstract

Marine phycoerythrin-containing cyanobacteria are major contributors to the overall productivity of the oceans. The present study indicates that the structural genes of the carbon assimilatory system are unusually arranged and possess a unique primary structure compared to previously studied cyanobacteria. Southern blot analyses of *Synechococcus* sp. strain WH7803 chromosomal DNA digests, using the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit gene from *Synechococcus* sp. strain PCC6301 as a heterologous probe, revealed the presence of a 6.4 kb *Hin*dIII fragment that was detectable at only low stringency. Three complete open reading frames (ORFs) were detected within this fragment. Two of these ORFs potentially encode the *Synechococcus* sp. strain WH7803 *rbcL* and *rbcS* genes. The third ORF, situated immediately upstream from *rbcL*, potentially encodes a homologue of the *ccmK* gene from *Synechococcus* sp. strain PCC7942. The deduced amino acid sequences of each of these ORFs are more similar to homologues among the β/γ purple bacteria than to existing cyanobacterial homologues and phylogenetic analysis of the Rubisco large and small subunit sequences confirmed an unexpected relationship to sequences from among the β/γ purple bacteria. This is the first instance in which the possibility has been considered that an operon encoding three genes involved in carbon fixation may have been laterally transferred from a purple bacterium. Analysis of mRNA extracted from cells grown under diel conditions indicated that *rbcL*, *rbcS* and *ccmK* were regulated at the transcriptional level; specifically Rubisco transcripts were highest during the midday period, decreased at later times during the light period and eventually reached a level where they were all but undetectable during the dark period. Primer extension analysis indicated that the *ccmK*, *rbcL* and *rbcS* genes were co-transcribed.

Introduction

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a pivotal enzyme in the pathway of carbon fixation of virtually all primary producers. This enzyme may thus play an important role in the modulation of CO_2 levels on a global scale and it is undoubtedly one of the most important and abundant enzymes in the biosphere [14]. Assessing the magnitude and variance of primary production in the marine environment is a major goal of biological oceanography. That the marine cyanobacteria make a significant contribution to total primary productivity, particularly in oligotrophic waters, is unquestioned [22]. In the current studies we have set out to investigate the transcriptional regulation of Rubisco in the marine cyanobacterium *Synechococcus* sp. strain WH7803, a unicellular phycoerythrin-containing bacterium. The marine cyanobacteria assigned to the genus *Synechococcus*, discovered in 1979, are abundant components of the picoplankton in both temperate and tropical oceans [21, 57]. Members of the *Synechococcus* sp. strain WH7803 serogroup can be prevalent in coastal

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U46156.

regions [5] while the closely related strain, WH8103, is probably more abundant in open ocean waters [19]. In spite of their apparent importance, virtually nothing is known regarding the control of CO_2 fixation in these cyanobacteria and so far the Rubisco operon has not been cloned from any of the representatives of this group of cyanobacteria. In this study we have attempted to elucidate the general features of the *Synechococcus* sp. strain WH7803 *rbc* operon and determine how this operon is controlled in response to light regimes. Comparable studies with natural populations of picoplankton indicate that there is a discrete regulatory response [39].

The present study indicated that the *Synechococcus* sp. strain WH7803 carbon fixation genes are organized and regulated in a unique fashion. In addition, phylogenetic analysis of the amino acid sequences reported here revealed unexpected relationships to sequences from among the purple bacteria.

Materials and methods

Bacterial strains, plasmids, phage and growth conditions

Synechococcus sp. strain WH7803 was grown in batch culture as described [49]. *Escherichia coli* JM107 [28] was grown in media as described by Maniatis *et al.* [28] and used for all plasmid constructions, preparations of phage, and plasmid DNA sequencing. All genomic DNA libraries were constructed in plasmid pK18 [43]. DNA sequencing was performed using either M13 mp18 for single-stranded templates or pK19/pK18 [43] for sequencing of double-stranded DNA.

DNA manipulations

Chromosomal DNA was isolated from *Synechococcus* sp. strain WH7803 [48] and plasmid isolations from *E. coli* were performed by the alkaline lysis method [3]. Digestion of plasmid and genomic DNA and ligations using T4 DNA ligase were performed by standard procedures [28]. Transformations were performed by the method of Chung *et al.* [10]. DNA fragments were purified from agarose gels using the GeneClean II Kit (Bio101, La Jolla, CA). DNA was transferred to nylon membranes (GeneScreen Plus, DuPont) by Southern blotting using procedures recommended by the manufacturer. Probes were labelled with [α-^{32}P]dCTP (DuPont) by random priming using a kit purchased from Amersham. Labelling was carried out according to the manufacturer's instructions. Hybridization was carried out as described in the GeneScreen Plus (DuPont) booklet. Wash stringencies are as described in Results. A partial genomic DNA library of *Synechococcus* sp. strain WH7803 was constructed using 6.0–7.5 kb *Hin*dIII chromosomal DNA fragments cloned into pK18. A 6.4 kb *Hin*dIII fragment was found to contain the genes of interest. Sequencing the ends of this fragment revealed the C-terminus of the *rbcS* gene. A 2.3 kb *Xho*I/*Hin*dIII fragment, presumed to contain the entire Rubisco gene operon, was subsequently subcloned and sequenced.

RNA extraction, Northern blotting and primer extension

Total RNA was extracted from exponentially growing cultures of *Synechococcus* sp. strain WH7803 as described [49]. Northern transfer and hybridization were performed as described [49] except that gels and loading buffer were made up using MOPS buffer [28] and RNA was transferred to nylon membranes (GeneScreen Plus). Hybridization and washes were carried out according to GeneScreen Plus protocols. Primer extensions were carried out as described [2].

DNA sequencing and computer analysis

The nucleotide sequence of the *Synechococcus* sp. strain WH7803 2.3 kb *Xho*I/*Hin*dIII fragment was determined by the dideoxy chain termination method [47] using a Sequenase kit purchased from USB. Most of the sequencing was performed by random subcloning into M13 mp18. Sequencing was completed using specific primers to sequence parts of the entire fragment cloned into pK18 or M13 mp18. The sequence was determined on both strands. Computer analyses of nucleic acid and protein sequences were carried out with the UWGCG software package [12]. ORFs contained in the 2.3 kb *Xho*I/*Hin*dIII fragment were identified with a *Synechococcus* sp. strain WH7803 codon usage table [6]. Multiple sequence alignments were performed with ClustalW [54]. Phylogenetic analyses were carried out with Phylip 3.5c [16].

Results

Cloning and sequence analysis of the Synechococcus *sp. strain WH7803 carbon fixation genes*

A 6.4 kb *Hin*dIII fragment of *Synechococcus* sp. strain WH7803 chromosomal DNA that hybridized, albeit at low stringency (6 × SCC at 55 °C), to the *Synechococcus* sp. strain PCC6301 *rbcL* probe, was cloned into pK18. A 2.3 kb *XhoI*/*Hin*dIII subclone was subsequently sequenced. The sequenced fragment was shown to potentially encode three proteins. These are the expected Rubisco large subunit and small subunit proteins (coordinates 470–1882 and 1945–2283 respectively) and an additional protein, coordinates 87–395, that shows significant similarity to a protein involved in the carbon concentrating mechanism of cyanobacteria, CcmK [23]. The arrangement, transcriptional orientation, sequence and deduced amino acid sequences are shown in Fig. 1. Two pairs of primers homologous to distinct N-terminal regions of the *ccmK* and *rbcL* genes were used for primer extension analysis. The *ccmK* primers only detected a single transcriptional start site upstream of the *ccmK* gene (Fig. 1B). Figure 1C shows the results of a primer extension analysis using one of the two primers homologous to the N-terminal sequence of the *ccmK* gene. No transciptional start sites were detected upstream of the *rbcL* gene. This observation indicated that the *ccmK*, *rbcL* genes were co-transcribed, an arrangement that, to our knowledge, is unique amongst all known bacteria containing *ccm* and *rbc* operons. The start site identified here does not appear to have −10 or −60 sequences that bear any resemblance to those previously identified, on the basis of sequence alignments alone [6], upstream of the *cpeB* genes of *Synechococcus* sp. strains WH7803 [34] and WH8020 [61] or the *Synechococcus* sp. strain WH7803 *pstS* gene [49]. Another interesting feature of the start site is that it is only 30 bases upstream from the *ccmK* translational initiation codon while transcription start sites previously identified for the *cpeB* and *pstS* gene of these organisms are > 100 bases upstream of the translational start site. Analysis of the DNA sequence did not reveal the presence of any strong terminator signals although putative hairpin loop structures were detected downstream of *rbcL* and *rbcS*.

The putative large subunit sequence was most closely related to the Form I type Rubisco large subunits, however, in each case sequences from among the β/γ purple bacteria showed significantly higher

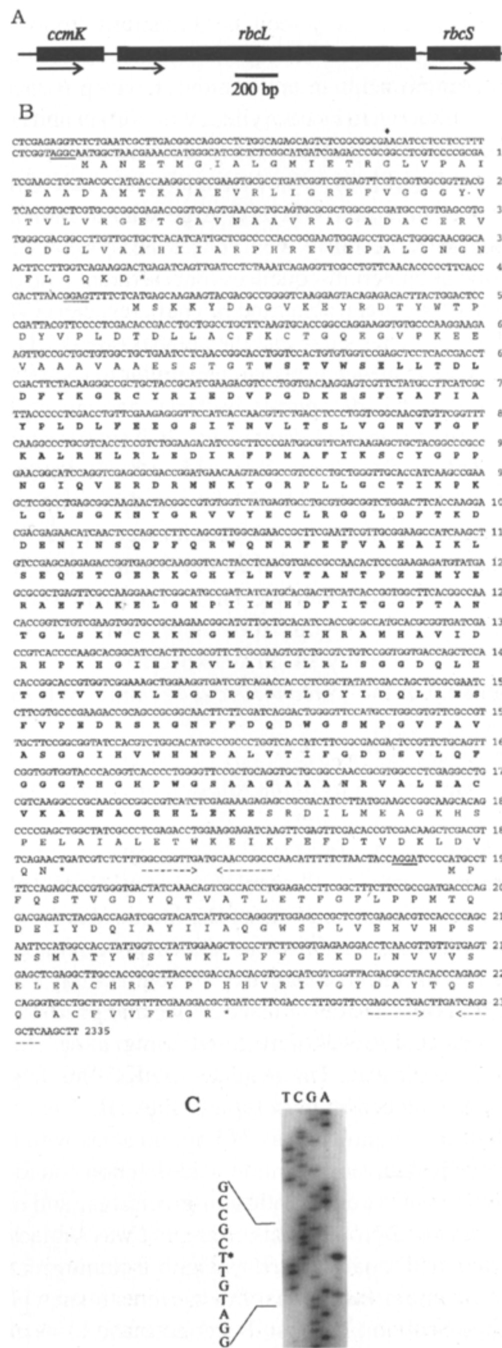

Figure 1. A. Arrangement and transciptional orientation of genes encoded by a 2335 bp fragment of *Synechococcus* sp. strain WH7803 DNA. B. DNA and deduced amino acid sequences of the 2335 bp fragment *Synechococcus* sp. strain WH7803 DNA. Putative ribosome-binding sites are underlined, hairpin-loop structures are indicated by dashed arrows and the transcriptional start site upstream of *ccmK* is identified with a ♦ above the sequence. The portion of the RbcL sequence in bold type was used in subsequent phylogenetic analyses. C. Primer extension studies indicating a single major transcription site upstream from *ccmK*. The sequence of the primer used in this analysis was 5′-TCATGCCGAGAGCGATGCC-3′ at coordinates 113–95 of the sequence.

similarity to the query sequences than did any of the complete cyanobacterial sequences present in the databases. Amino acids in and around the loop 6 region have been shown to be catalytically important and contribute to the ability of Rubisco to discriminate between CO_2 and O_2 [8, 20, 38, 44], analysis of the *Synechococcus* sp. strain WH7803 large subunit indicates this region to be fairly well conserved, compared to other cyanobacterial sequences, but different from the enzyme of nongreen algae and β/γ purple bacteria [44]. Database searches show that the putative small subunit amino acid sequence is also more similar to sequences from among the β/γ purple bacteria than any of the known cyanobacterial sequences. It is most similar to the *Thiobacillus ferrooxidans* (54.1% identity) and *Thiobacillus neopolitanus* (52.3% identity) sequences while the most similar cyanobacterial sequence is from *Anabaena* sp. strain PCC7120 (41.7% identity). The greater identity of both the large and small subunit sequences to homologous from the β/γ purple bacteria raised the possibility of an unexpected evolutionary relationship of the *Synechococcus* sp. strain WH7803 Rubisco.

Phylogenetic analysis of the Rubisco large and small subunit protein sequences

During the course of our study, a partial amino acid sequence, 375 amino acids, of the large subunit from *Prochlorococcus marinus* was shown to be related to β/γ purple bacterial sequences [50]. In this study RbcL sequences from a variety of sources were aligned with a 375 amino acid stretch of the *Synechococcus* sp. strain WH7803 sequence. The residues used in this alignment are indicated in bold type in Fig. 1B. The total length of the alignment was 375 amino acids with the only gap being a single amino acid deletion found in all members of the eukaryotic non-green algae and α/β purple bacterial groups. This alignment was subjected to phylogenetic analysis by the neighbor-joining method with Kimura evolutionary distance estimates [46] using the Seqboot, Protdist, Neighbour and Consense programs from Phylip v. 3.5 [16]. This analysis produced a phylogenetic tree that places the *Synechococcus* sp. strain WH7803 Rubisco large subunit protein within a group containing members of the β/γ purple bacteria and a single member of the α purple bacteria, *Nitrobacter vulgaris* (Fig. 2A). Indeed, the sequence most closely related to the *Synechococcus* sp. strain WH7803 large subunit is that from *P. marinus*, while both of these sequences are most closely related to the deduced amino acid sequence of the second Rubisco large subunit gene of *Chromatium vinosum*, a gene that appears to be barely expressed under standard photoautotrophic growth conditions in this bacterium [25]. Bootstrap analysis of this data indicated a high degree of confidence in the tree topology, particularly over the region of interest. Parsimony and maximum likelihood analyses of the same data set produced a similar tree with an identical topology in the region of interest (data not shown). The alignment used in the large subunit phylogenetic analysis presented here (Fig. 2A) was constrained to the length of the *P. marinus* sequence. If this sequence was omitted from the analysis and as large a portion of the *Synechococcus* sp. strain WH7803 large subunit as possible used in the analysis, the tree generated was almost identical to that shown in Fig. 2A, with the *Synechococcus* sp. strain WH7803 sequence being most closely related to that of the *C. vinosum* large subunit 2 sequence (data not shown). In both cases bootstrap analysis indicated a high degree of confidence in the branching in this region of the tree. Phylogenetic analysis of the *Synechococcus* sp. strain WH7803 small subunit protein also placed this sequence within the β/γ purple bacteria radiation (Fig. 2B). An alignment length of 95 amino acids was used; the residues used in the phylogenetic analysis are boxed in Fig. 3. The *Synechococcus* sp. strain WH7803 sequence is, like that large subunit sequence, most closely related to the sequences of the *C. vinosum* 2 and *Hydrogenovibrio marinus* 2 small subunits (no small subunit sequence is available for *P. marinus*). Again parsimony and maximum likelihood analyses of this data set support the results of the analysis shown in Fig. 2B. Although the bootstrap values of several nodes in Fig. 2B are somewhat low the results of this analysis support the placement of the *Synechococcus* sp. strain WH7803 large subunit protein within this group. Of particular interest is the observation that the *T. neopolitanus* small subunit is not particularly closely related to the *Synechococcus* sp. strain WH7803 small subunit (see below). The general features of alignments of the RbcS sequences has been discussed in some detail [32]. The alignment shown here (Fig. 3), does, however, show some noteworthy additions and differences to that of Morden and Golden [32]. In particular the *Synechococcus* sp. strain WH7803 sequence shares an extended N-terminal region with that from the *C. vinosum* 2 sequence. Several of the other β/γ purple bacteria also have a somewhat extended N-terminal region when compared to the other sequence groups in this ana-

lysis. Our alignment differs from that of Morden and Golden [32] in the sequence gaps from position 15–23 of the alignment. We find that a gap from position 15–20 of the *Synechococcus* sp. strain WH7803 sequence is shared with all the cyanobacterial sequences, the *T. neopolitanus* and *N. vulgaris* sequences, and RbcS 2 sequences from *C. vinosum* and *H. marinus*, but is not present in the other β/γ purple bacteria. Otherwise our alignment is similar to that of Morden and Golden [32] except for the way the gaps in the center of the sequence, 55–76, were treated. Gaps, except that at 133–134, were excluded from the phylogenetic analysis.

The Synechococcus *sp. strain WH7803 CcmK homologue*

The inorganic carbon-concentrating mechanism (CCM) endows cyanobacteria with the ability to adapt to a wide range of ambient CO_2 concentrations. Mutants with a growth requirement for high CO_2 concentrations are, by definition, defective in some aspect of their carbon-concentrating mechanism [42]. The carbon-concentrating mechanism has best been studied in the cyanobacterial strains *Synechococcus* sp. strain PCC7942 [17, 29, 41, 42] and *Synechocystis* sp. strain PCC6803 [35, 36], both freshwater cyanobacteria. Little is known of the function of the *ccm* genes in these strains although many high-CO_2-requiring mutants have the common feature that carboxysomes are either aberrant or completely absent [23]. Although polyhedral bodies, presumably carboxysomes, have been observed in *Synechococcus* sp. strain WH7803 [26], Karagouni *et al.* [24] found no evidence for a carbon-concentrating mechanism in *Synechococcus* sp. strain WH7803, WH8110, and WH8018 and speculated that the relatively slow-growing marine *Synechococcus* species would never become rate-limited with respect to available bicarbonate and thus would be under no selective pressure to maintain a carbon-concentrating mechanism. Nonetheless, the deduced amino acid sequence immediately upstream from the *rbcLS* sequences was found to be similar to the CcmK sequences from *Synechococcus* sp. strain PCC7942 (53.9% identity) and *Synechococcus* sp. strain PCC6301 (36.5% identity). However, the sequence(s) most closely related to the *Synechococcus* sp. strain WH7803 sequence were β purple bacterial sequence(s) from *T. neopolitanus*. Immediately downstream of the *T. neopolitanus rbc* operon is a series of three repeats, each potentially encoding a *ccmK* homologue [15]. One of these genes, *csoS1A*, is known to be transcribed. CcmK from *Synechococcus* sp. strain WH7803 is 88.4% identical to the csoS1A polypeptide expressed in *T. neopolitanus*. English *et al.* [15] suggest that the csoS1A polypeptide is a component of the carboxysome shell. Recently a *ccmK*-like sequence, *pduA*, was identified in *Salmonella typhimurium* [7]. Clearly this organism does not have carboxysomes and the authors speculate that the PduA protein (58.8% identical to the sequence reported here) may in some way protect a *S. typhimurium* propanediol dehydratase complex from oxygen. The cyanobacterial sequences from *Synechococcus* sp. strain PCC7942 and *Synechococcus* sp. strain PCC6301 show considerably less similarity to the *Synechococcus* sp. strain WH7803 protein than the CsoS1 and PduA sequences. Since the *ccm* genes are, to some extent, clustered in cyanobacteria it was surprising that subsequent studies failed to identify any other *ccm*-like sequences in a region spanning 4 kb upstream of *ccmK* (data not shown).

Distribution of related sequences

We have performed Southern blot analyses using probes derived from the *Synechococcus* sp. strain WH7803 *rbcL* and *ccmK* sequences to interrogate chromosomal DNA digests from a variety of prokaryotic sources including representitives of all the major groups of prokaryotic form I Rubisco. At low stringencies, homologues of the *rbcL* gene could be detected in all of the DNAs examined with suitably long exposures, except that from *P. marinus* (Fig. 4A). The lack of detectable hybridization to the *P. marinus* DNA is somewhat surprising. A possible explanation for this is that although the *P. marinus* Rubisco DNA sequence is 71% identical to the *rbcL* probe derived from *Synechococcus* sp. strain WH7803, virtually all of the differences are accounted for by third base degeneracies with the result that the longest stretch of identities is only 14 bp. Such short stretches of identity may not have been detected even when low stringency washes were performed. At high stringency only fragments present in marine phycoerythrin-containing cyanobacteria were detected (Fig. 4B). *Synechococcus* sp. strains WH7803 and WH7805 showed the same restriction fragment length polymorphism (RFLP), as did *Synechococcus* sp. strains WH8102 and WH8103. The same banding pattern was also observed in blots probed at high stringency with a *Synechococcus* sp. strain WH7803 *ccmK* probe (Fig. 4C). All of these WH strains are representatives of the mar-

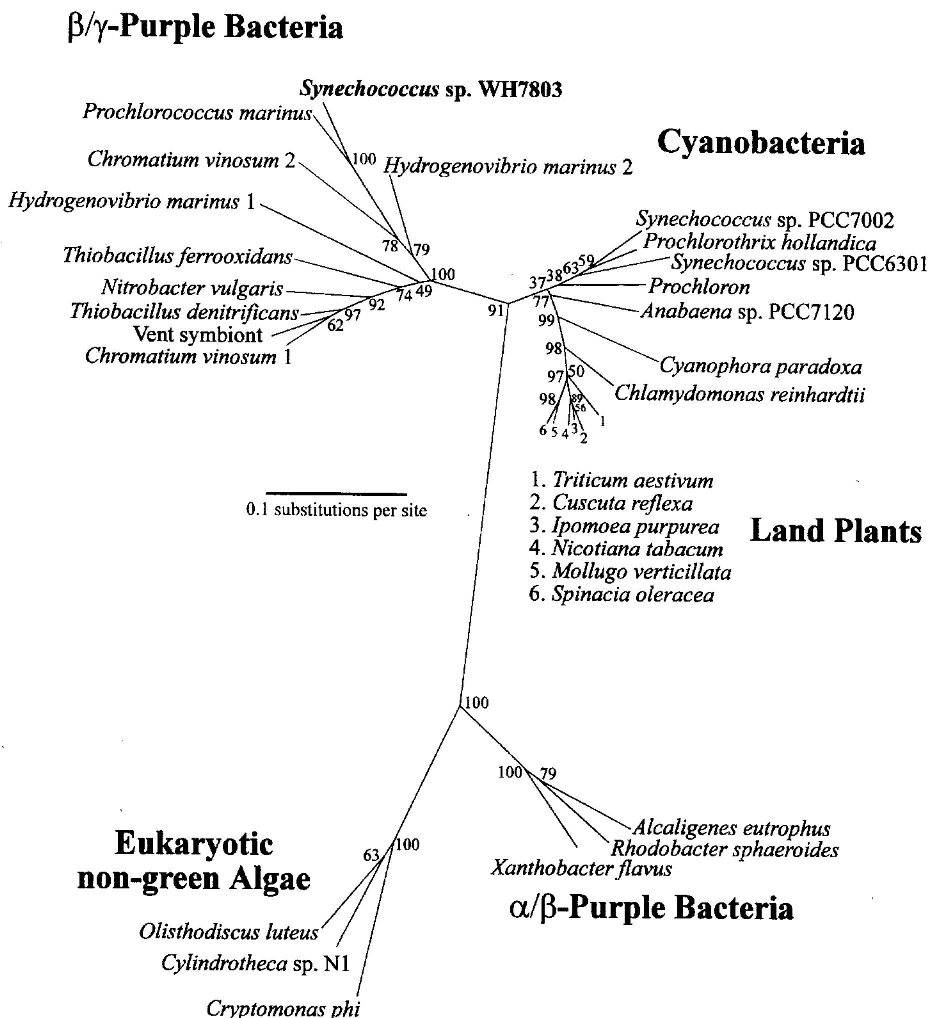

Figure 2. Molecular phylogenetic tree derived from selected Rubisco large and small subunit amino acid sequences. Multiple sequence alignments were performed using ClustalW, the small subunit alignment was further adjusted manually. Tree topology and evolutionary distance estimations were performed by the neighbor-joining method using Kimura distances (Phylip 3.5). These trees are unrooted. Bootstrap values, calculated from 1000 replicates using the Seqboot, Protdist, Neighbor and Consense programs of Phylip 3.5 are indicated at the nodes of the tree and are expressed as percentages. A. Phylogenetic analysis of large subunit sequences. Accession numbers of Rubisco large subunit (RbcL or CbbL) homologues: *Alcaligenes eutrophus*, P09657; *Anabaena* sp. strain PCC 7120, P00879; *Chlamydomonas reinhardtii*, P00877; *Chromatium vinosum* 1, P22849; *Chromatium vinosum* 2, P22859; *Cryptomonas phi*, P14957; *Cuscuta reflexa*, P30401; *Cyanophora paradoxa*, P24312; *Cylindrotheca* sp. strain N1, P24673; *Hydrogenovibrio marinus* 1, D43621 (gb); *Hydrogenovibrio marinus* 2, D43622 (gb); *Ipomoea purpurea*, P28260; *Mollugo verticillata*, P25832; *Nicotiana tabacum*, P00876; *Nitrobacter vulgaris*, L22885 (gb); *Olisthodiscus luteus*, P14959; *Prochlorothrix hollandica*, P27568; *Prochlorococcus marinus*, D21833 (gb); *Prochloron* sp., D21834 (gb); *Rhodobacter sphaeroides*, P27997; *Spinacia oleracea*, P00875; *Synechococcus* sp. strain PCC 6301, P00880; *Synechococcus* sp. strain PCC 7002, D13971 (gb) *Triticum aestivum*, P11383; *Thiobacillus denitrificans*, L42940 (gb), *Thiobacillus ferrooxidans*, P28895; Vent symbiont (potential *Thiobacillus*), P24672; *Xanthobacter flavus*, P23011. B. Phylogenetic analysis of small subunit sequences. Accession numbers of Rubisco small subunit (RbcS or CbbS) homologues; *Alcaligenes eutrophus*, M17744 (gb); *Anabaena* sp. strain PCC7120, P06514; *Arabidopsis thaliana*, P10795; *Chlamydomonas reinhardtii* 1, P00873; *Chlamydomonas reinhardtii* 2, P08475; *Chromatium vinosum* 1, P22850; *Chromatium vinosum* 2, P22860; *Cryptomonas phi*, P14960; *Cyanophora paradoxa*, P18062; *Cylindrotheca* sp. N1, M59080 (gb); *Hydrogenovibrio marinus* 1, D43621 (gb); *Hydrogenovibrio marinus* 2, D43622 (gb); *Nicotiana tabacum*, P00866; *N. vulgaris*, L22885 (gb); *Olisthodiscus luteus*, M24288 (gb); *Prochlorothrix hollandica*, X57359 (gb); *Rhodobacter sphaeroides*, P27998; *Spinacia oleracea*, P00870; *Synechococcus* sp. strain PCC6301, X03222 (gb); *Synechococcus* sp. strain PCC7002, D13971 (gb); *Thiobacillus denitrificans*, L42920 (gb); *Thiobacillus ferrooxidans*, P28896; *Thiobacillus nepolitanus*, L38256 (gb); Vent symbiont (potential *Thiobacillus*), P24682; *Triticum aestivum* 1, P00871; *Triticum aestivum* 2, P26667; *Xanthobacter flavus*, P23012. All sequences are from the SwissProt database with the exception of those marked (gb) GenBank/EMBL.

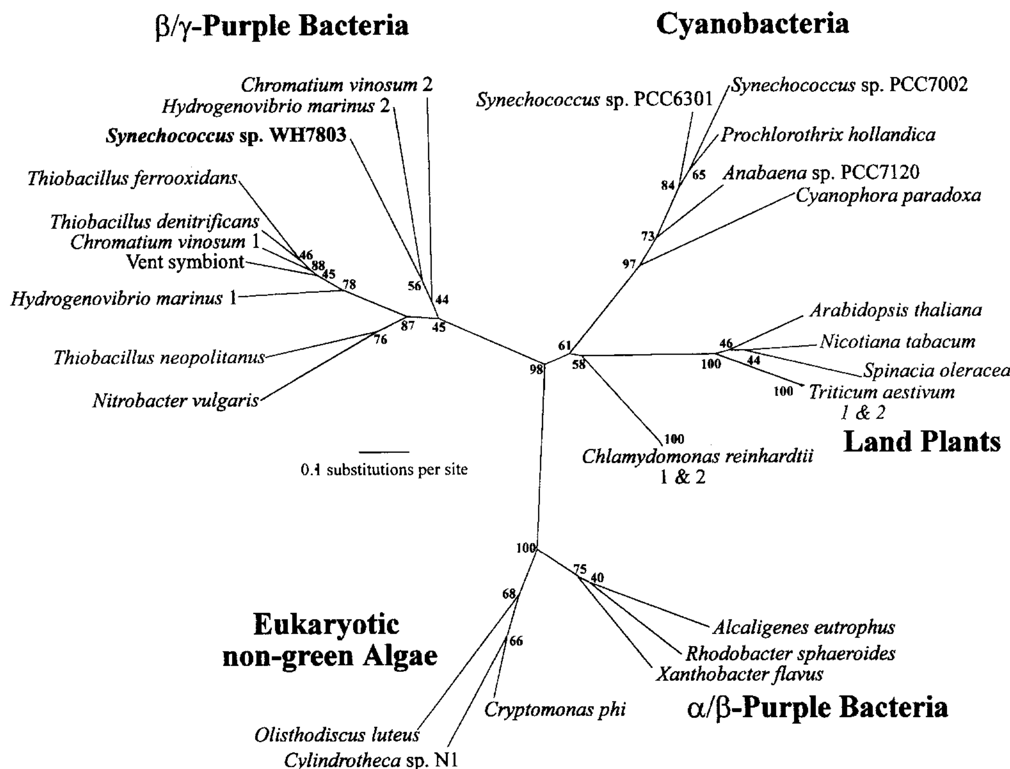

Figure 2. Continued.

ine cyanobacterial group A cluster [58]. The results presented here demonstrate that the sequences of both the *rbc*L and *ccmK* homologues in these organisms are > 90% identical at the DNA sequence level and the RFLP similarities could even suggest a similar arrangement of these genes in these organisms. These species are representatives of both coastal and open ocean phycoerythrin-containing cyanobacteria. Thus, it seems possible that a large percentage of carbon-fixing activity in both coastal and open ocean waters is accomplished using this form of Rubisco.

Transcription of rbcL *in* Synechococcus *sp. strain WH7803*

In initial experiments, RNA was prepared from *Synechococcus* sp. strain WH7803 grown under constant light conditions. Northern blots of purified RNA were probed with the 0.78 kb *Eco*RI/*Bam*HI fragment. This fragment hybridized to three major transcripts of 2.3, 1.9 and 1.3 kb (data not shown). Transcriptional control of the Rubisco operon was then investigated in cultures grown in artificial sea water medium under a 14/10 h light/dark cycle at a light intensity of 25 μM quanta m^{-2} s^{-1}; cultures were grown with constant stirring and aeration. There was a distinct variation in transcript levels as a function of time (Fig. 5). In these experiments the 'daylight' period started at 06:00 and ended at 20:00. Transcript levels accumulated fairly rapidly and maximal levels of transcript were detected about 6 h after illumination was started. Rubisco transcript levels then gradually tapered off and there was a distinct 'anticipation' of the dark phase until transcripts were barely detectable in the late stage of the dark period. At the beginning of the next 'day' the same pattern of transcript accumulation was observed. Similar results to those shown in Figure 5 were obtained in two repeats of this experiment. A circidian rhythm in cell division has been shown to exist in *Synechococcus* sp. strain WH7803 [51] and the cyclical nature of the variations in transcript levels detected here are in keeping with such a circadian rhythm.

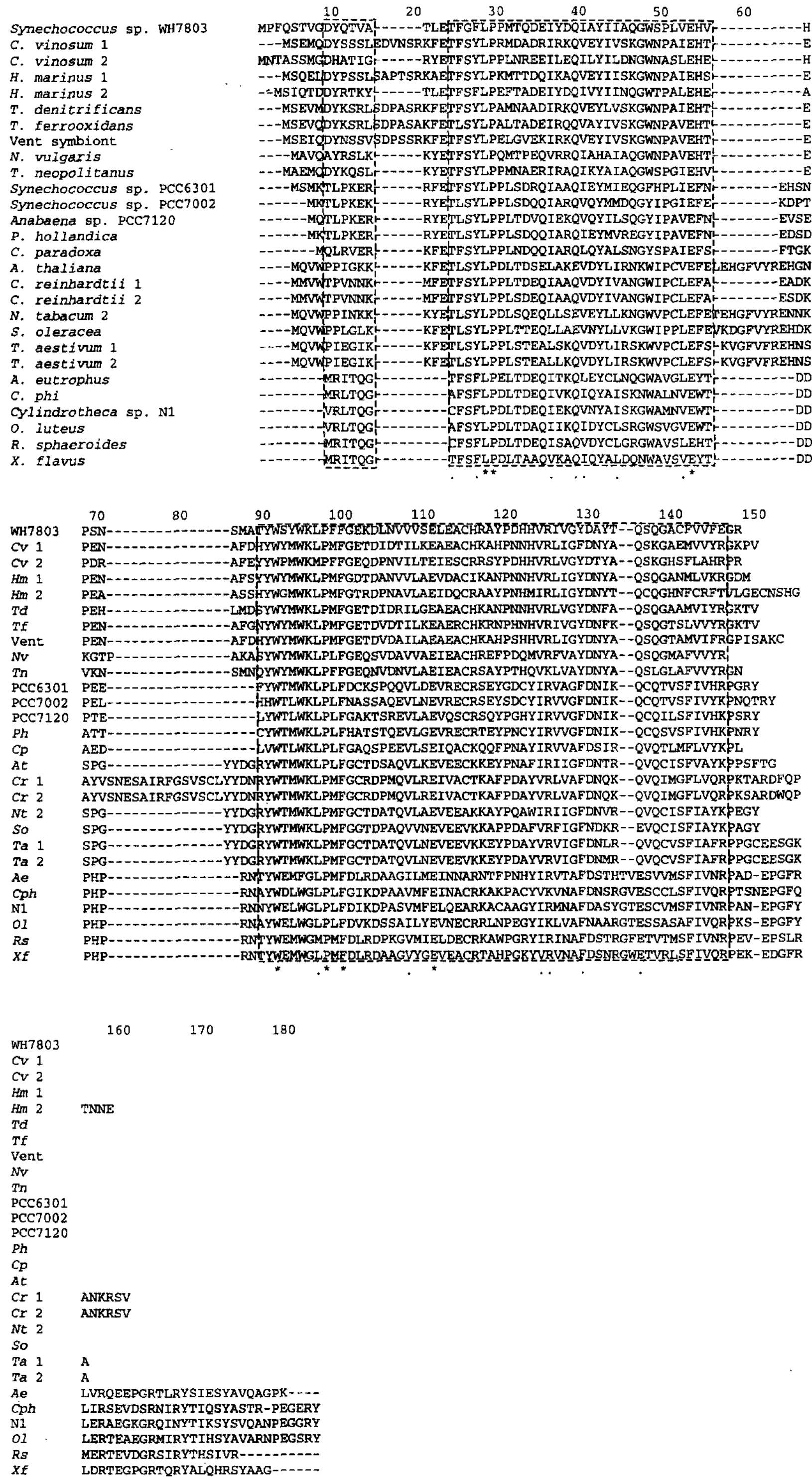

Figure 3. Alignment of Rubisco small subunit sequences. This multiple sequence alignment was performed using ClustalW and then further adjusted manually. Identical residues present in all sequences are marked with a * while conserved residues are marked with a •. Boxed residues were used to perform phylogenetic analyses.

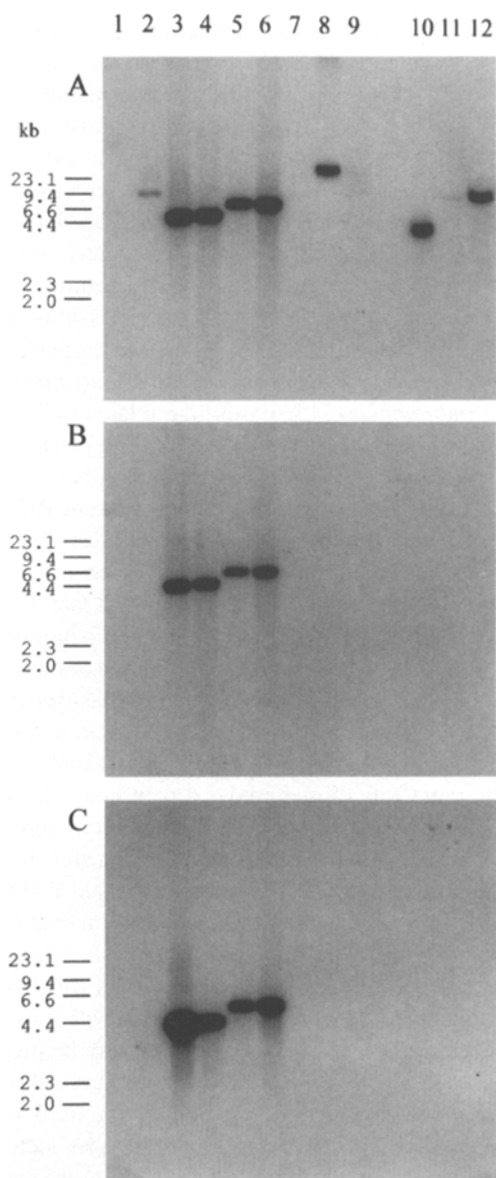

Figure 4. Distribution of *rbcL* and *ccmK* homologues in a selection of bacteria. DNA in lanes 1–8 were digested with *Bam*HI and lanes 9–12 with *Eco*RI. 2.5 μg DNA was loaded per lane. Lanes: 1, *Rhodobacter sphaeroides*; 2, *Thiobacillus ferroxidans*; 3, *Synechococcus* sp. strain WH7803; 4, *Synechococcus* sp. strain WH7805; 5, *Synechococcus* sp. strain WH8102; 6, *Synechococcus* sp. strain WH8103; 7, *Prochlorococcus marinus*; 8, *Synechococcus* sp. strain PCC7942; 9, *Anabaena* sp. strain PCC7120; 10, *Synechocystis* sp. strain PCC6803; 11, *Anabaena variabilis*; 12, *Anabaena* sp. strain CA. A. Southern blot analysis of digested chromosomal DNA probed with a 615 bp *Eco*RI/*Xho*I fragment encoding the C-terminal portion of the RbcL protein. Washes 2× 1 h in 2 × SSC at 55 °C. B. Similar to A, except that washes were performed in 0.1 × SSC at 65 °C. Exposure time was normalized to be equivalent to that in A. C, Repeat Southern blot probed with a 283 bp *Xho*I/*Nar*I fragment encoding the N-terminal portion of the CcmK protein. Washes were performed in 0.1 × SSC at 65 °C.

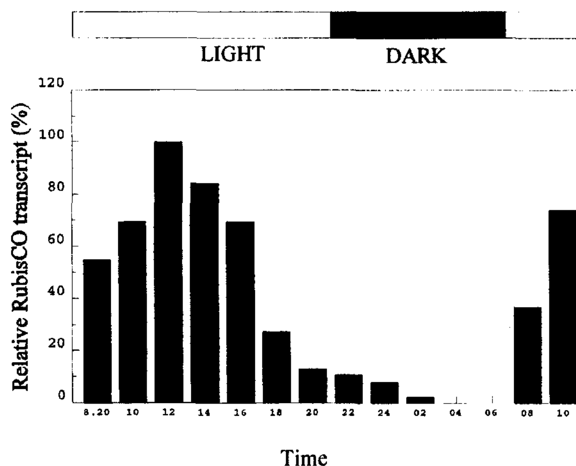

Figure 5. rbcL transcript accumulation in *Synechococcus* sp. strain WH7803 grown under a 14/10 h light/dark cycle. *rbcL* mRNA levels are expressed as a percentage of maximum levels. 10 μg RNA was loaded per lane. The Northern blot was probed with a 0.78 kb *Bam*HI/*Eco*RI fragment containing the entire C-terminal end of the *rbcL*-coding sequence. Hybridizing transcripts were quantified using an Instantimager (Packard Instrument Company).

Discussion

This is the first report of the sequence and regulation of the carbon fixation genes from a marine phycoerythrin-containing cyanobacterium. These organisms play a significant role in marine, and hence global, primary productivity [22]. While studies of marine cyanobacterial carbon fixation and the regulation of carbon fixation genes have been carried out, respectively, by analysis of bicarbonate fixation in size-fractionated waters [40] and the use of heterologous probes to detect Rubisco transcripts in the water column [39], an underlying assumption of these studies is that carbon fixation is accomplished by typical cyanobacterial-type Rubisco enzymes. In this work we set out to study the regulation of carbon fixation genes in *Synechococcus* sp. strain WH7803. As a first step, we cloned and sequenced a 2.3 kb fragment of *Synechococcus* sp. strain WH7803 chromosomal DNA that was known to contain the *rbc* genes. This fragment was subsequently found to potentially encode three genes, the expected *rbcL* and *rbcS* genes and a homologue of the *ccmK* gene. The deduced amino acid sequences of both the large and small subunit Rubisco proteins were most similar to Rubisco sequences found among the β/γ purple bacteria. Subsequent phylogenetic analysis using a broad selection of form I large and small subunit sequences strongly indicated that the sequence

reported here does not fall within the cyanobacterial radiation but rather within a group containing the β/γ purple bacteria. The phylogenetic analysis of the small subunit sequences provides further evidence that *Synechococcus* sp. strain WH7803 contains a proteobacterial type Rubisco. The 'β/γ purple bacterial' and 'cyanobacterial' Rubisco proteins have been classified in types IA and IB and have distinct biochemical properties [53].

We think it unlikely that the sequence reported here came from an undetected contaminant for the following reasons: chromosomal DNA from three independently maintained stocks of *Synechococcus* sp. strain WH7803 all contain the same hybridizing fragment; a *Synechococcus* sp. strain WH7803 codon usage table [6] accurately detects the ORFs reported here; closely related strains contain closely related, but not identical (data not shown) sequences; and a single hybridizing fragment is detected in *Synechococcus* sp. strain WH7803 DNA probed at low stringency with either the *Synechococcus* sp. strain WH7803 or the *Synechococcus* sp. strain PCC6301 *rbcL* gene.

The relationship of the *Synechococcus* sp. strain WH7803 Rubisco large subunit to the β/γ purple bacteria is similar to that observed for *P. marinus*. The *P. marinus* 16S rRNA and *rpo*C1 DNA sequences have been the subject of phylogenetic analyses which have placed *P. marinus* within the cyanobacterial radiation [37, 55]. Similarly, 16S rRNA sequences from *Synechococcus* sp. strain WH7805 and WH8103, strains closely related to *Synechococcus* sp. strain WH7803, also place these strains within the cyanobacterial radiation [55]. Shimada *et al.* [50] proposed *P. marinus* to be the most primitive oxygenic prokaryote. This proposal is based on the relationship of *P. marinus* Rubisco to the γ purple bacteria and the fact that *Prochlorococcus* contains divinyl chlorophylls *a* and *b* and a chlorophyll-*c*-like pigment, in keeping with the proposed pigment content of the first photosynthetic organism [27]. In contrast to Shimada *et al.* [50], Urbach *et al.* [55] suggest that the shallowness of the *Prochlorococcus* cluster, in 16S rRNA comparisons, indicates a particularly recent origin for this organism's pigment phenotype. Larkum [27] has suggested that the phycourobilin chromophore present in *Synechococcus* sp. strain WH7803 was added to phycoerythrin as a final evolutionary development and recent studies of the evolution of phycobiliprotein sequences [1] support this view. Thus it would appear that *Synechococcus* sp. strain WH7803 is a relatively modern cyanobacterium and this in turn would seem to indicate that *P. marinus*, which has now been shown to be closely related to *Synechococcus* on the basis of 16S rRNA [55] and Rubisco amino acid sequences (this paper), is also a relatively modern organism. If this is indeed the case, the suggestion of a recent origin for the *Prochlorococcus* pigment phenotype would be supported and it seems likely that both *Synechococcus* sp. strain WH7803 and *P. marinus* must have acquired these Rubisco genes from a purple bacterium.

Recently several startling discoveries have been made regarding Rubisco phylogenies. In particular the Rubisco genes from the dinoflagellates *Gonyaulax* [33] and *Symbiodinium* [45, 59, 60] are related to Form II Rubisco which had previously only been observed in proteobacteria [53]. It is also well known that the non-green algae probably acquired their current Rubisco genes from a member of the α/β purple bacterial group [4, 56] while green algae and plant Rubisco group with the cyanobacteria [30, 31]. It is likely that lateral gene transfer of Rubisco genes has taken place a number of times and/or that an ancestral gene duplication of the form I Rubisco was followed by differential loss of one of the operons [11, 30]. Whatever the case, it is quite clear that phylogenies based on Rubisco analysis are incongruent with those based on other molecules such as 16S rRNA and hsp60. The observations reported here suggest yet another lateral gene transfer. In this case it appears that *Synechococcus* sp. strain WH7803 has acquired three genes involved in carbon fixation as an operon from a purple bacterium, a possible event that has not previously been reported. If this is true one might expect that a proteobacterium with a similar arrangement of carbon fixation genes may be present in the environment. The quite startling degree of identity between the CcmK sequence reported here to the *T. neopolitanus* csoS1A polypeptide (88.4% identity) might, on its own suggest *Thiobacillus* sp. as the source of the genes described here. However, phylogenies presented here do not strongly support this scenario. Nonetheless, the *T. neopolitanus* RbcS sequence does share a number of features with the *Synechococcus* sp. strain WH7803 sequence including the sequence gap between position 15 and 20 (Fig. 3). The *T. neopolitanus* RbcL sequence should be available soon and will be invaluable in further examining the relationship between these genes.

The CcmK protein is thought to be a major component of the carboxysome shell [15], an important component of the carbon concentrating mechanism [23]. Although carboxysome-like structures are present in *Synechococcus* sp. strain WH7803, the role of a CcmK

homologue in *Synechococcus* sp. strain WH7803 is unclear since a previous report did not find any evidence for a carbon-concentrating mechanism in this and several related *Synechococcus* sp. [24]. Chen *et al.* [7] have identified a gene, *pduA*, in *S. typhimurium* that potentially encodes a homologue of CcmK and speculated that the PduA protein may be involved in the assembly of a complex of Pdu enzymes and protection of these enzymes from oxygen damage. These observations combined with the sequence data presented here may indicate that (a) a complete carbon-concentrating mechanisms is not present in *Synechococcus* sp. strain WH7803 (b) that the CcmK protein is not necessarily a component of a carboxysome-like structure.

Hybridization studies using probes derived from the *rbcL* and *ccmK* sequences revealed that all the members of the marine cyanobacterial group A cluster examined here contain sequences that are $> 90\%$ identical to the probes used. These results contradict previous DNA polymorphism studies in which it was suggested that *Synechococcus* sp. strain WH7803 was no more closely related to *Synechococcus* sp. strain WH7805 than to freshwater cyanobacterial strains (e.g. *Synechococcus* sp. strain PCC6301) [13, 62].

Primer extension analyses indicated that there is a single transcriptional start site upstream of the *ccmK* gene. Thus it would seem that the three genes reported here are part of an operon, a gene arrangement that is unique for photosynthetic prokaryotes [18, 52]. Further transcriptional analysis using an *rbcL* specific probe revealed the presence of three major hybridizing fragments of 2.3, 1.9 and 1.3 kb (data not shown). In *Synechococcus* sp. strain WH7803, at least one of the transcripts would be expected to code for the three gene products. The pattern of transcription during a 14/10 h light/dark cycle was found to be similar to that observed in *Synechococcus* sp. strain RF-1 [9] with transcripts accumulating rapidly at the start of the photoperiod, reaching a peak after about 6 h and then gradually declining through the rest of the photoperiod and dark period until transcripts were almost undetectable after 6–8 h of darkness. Analysis of the diel variation of *rbcL* expression in natural waters also shows a discrete regulatory response to light with transcript levels highest at about noon, but increases in transcript levels were not as dramatic as those shown here [39].

In conclusion, the *ccmK*, *rbcL* and *rbcS* genes have been cloned from the marine phycoerythrin-containing cyanobacterium *Synechococcus* sp. strain WH7803. Deduced amino acid sequences from each of these genes revealed unexpected similarities to homologues of β/γ purple bacteria. Subsequent phylogenetic analysis also places the Rubisco large and small subunit sequences in close proximity to the β/γ purple bacteria sequences and not within the cyanobacterial radiation. The *ccmK* and *rbcL* genes appear to be cotranscribed, a so far unique phenomenon in photosynthetic organisms in which a *ccmK* homologue has been identified. Southern blot analyses indicate that these genes are highly conserved among marine phycoerythrin-containing cyanobacteria and it is quite possible that the arrangements of these genes in these strains will be similar. Finally transcriptional analyses and diel studies indicate that transcription is light-regulated.

Acknowledgments

We thank Nick Mann, John Waterbury and Bianca Brahamsha for generously providing us with strains; Scott Pichard and John Paul for *P. marinus* DNA; Chuck Daniels for the use of his Instantimager and Norman Pace and Charles Delwiche for suggestions regarding this manuscript. This work was supported by Grants OCE-9218517 and DE-FG02-93ER61700 from the National Science Foundation and Department of Energy, respectively.

References

1. Apt KE, Collier JL, Grossman AR: Evolution of the phycobiliproteins. J Mol Biol 248: 79–96 (1995).
2. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K: Current Protocols in Molecular Biology. John Wiley, Massachusetts (1995).
3. Birnboim HC, Doly J: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl Acids Res 7: 1513–1517 (1979).
4. Boczar BA, Delaney TP, Cattolico RA: Gene for the ribulose-1,5-bisphosphate carboxylase small subunit protein of the marine chromophyte *Olisthodiscus luteus* is similar to that of a chemoautotrophic bacterium. Proc Natl Acad Sci USA 86: 4996-4999 (1989).
5. Carpenter EJ, Campbell L: Diel patterns of cell division and growth rates of *Synechococcus* spp. in Long Island Sound. Mar Ecol Prog Ser 47: 179–183 (1988).
6. Carr NG, Mann NH: The oceanic cyanobacterial picoplankton. In: Bryant DA (ed) The Molecular Biology of the Cyanobacteria, pp. 27–48. Kluwer Academic Publishers, Dordrecht, Netherlands (1994).
7. Chen P, Andersson DI, Roth JR: The control region of the pdu/cob regulon in *Salmonella typhimurium*. J Bact 176: 5475–5482 (1994).
8. Chen Z, Spreitzer RJ: Chloroplast intragenic suppression enhances the low CO_2/O_2 specificity of mutant ribulose-

bisphosphate carboxylase/oxygenase. J Biol Chem 264: 3051–3053 (1989).

9. Chow T-J, Tabita FR: Reciprocal light-dark transcriptional control of *nif* and *rbc* expression and light-dependent posttranslational control of nitrogenase activity in *Synechococcus* sp. strain RF-1. J Bact 176: 6281–6285 (1994).
10. Chung CT, Niemela SL, Miller RH: One step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci USA 86: 2172–2175 (1989).
11. Delwiche CF, Palmer JD: Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plastids. Mol Biol Evol 13: 873–882 (1996).
12. Devereux J, Haeberli P, Smithies O: A comprehensive set of sequence analysis programs for the VAX. Nucl Acids Res 12: 387–395 (1984).
13. Douglas SE, Carr NG: Examination of genetic relatedness of marine *Synechococcus* spp. using restriction fragment length polymorphisms. Appl Environ Microbiol 54: 3071–3078 (1988).
14. Ellis RJ: The most abundant protein in the world. Trends Biochem Sci 4: 241–244 (1979).
15. English RS, Lorbach SC, Qin X, Shively JM: Isolation and characterization of a carboxysome shell gene from *Thiobacillus neapolitanus*. Mol Microbiol 12: 647–654 (1994).
16. Felsenstein J: PHYLIP (Phylogeny Inference Package). Department of Genetics, University of Washington, Seattle (1993).
17. Friedberg D, Kaplan A, Ariel R, Kessel M, Seijffers J: The 5′ flanking region of the gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase is crucial for growth of the cyanobacterium *Synechococcus* sp. strain PCC 7942 at the level of CO_2 in air. J Bact 171: 6069–6076 (1989).
18. Gibson JL: Genetic analysis of CO_2 fixation genes. In: Blankenship RE, Madigan MT, Bauer CE (eds) Anoxygenic Photosynthetic Bacteria, pp. 1107–1124. Kluwer Academic Publishers, Dordrecht, Netherlands (1995).
19. Glover HE, Prézelin BB, Campbell L, Wyman M: Pico- and ultraplankton Sargasso Sea communities: variability and comparative distributions of *Synechococcus* spp. and algae. Mar Ecol Prog Ser 49: 127–139 (1988).
20. Gutteridge S, Rhoades DF, Herrmann C: Site-specific mutations in a loop region of the C-terminal domain of the large subunit of ribulose bisphosphate carboxylase/oxygenase that influence substrate partitioning. J Biol Chem 268: 7818–7824 (1993).
21. Johnson P, Sieburth J: Chroococcoid cyanobacteria in the sea: a ubiquitous and diverse phototrophic biomass. Limnol Oceanogr 24: 928–935 (1979).
22. Joint IR: Physiological ecology of picoplankton in various oceanographic provinces. Can Bull Fish Aquat Sci 214: 287–309 (1986).
23. Kaplan A, Schwarz R, Lieman-Hurwitz J: Reinhold L: Physiological and molecular studies on the response of cyanobacteria to changes in ambient inorganic carbon concentration. In: Bryant DA (ed) The Molecular Biology of the Cyanobacteria, pp. 469–485. Kluwer Academic Publishers, Dordrecht, Netherlands (1994).
24. Karagouni AD, Bloye SA, Carr NG: The presence and absence of inorganic carbon concentrating systems in unicellular cyanobacteria. FEMS Microbiol Lett 68: 137–142 (1990).
25. Kobayashi H, Viale AM, Takabe T, Akazawa T, Wada K, Shinozaki K, Kobayashi K, Sugiura M: Sequence and expression of genes encoding the large and small subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase from *Chromatium vinosum*. Gene 97: 55–62 (1991).
26. Kursar TA, Swift H, Alberte RS: Morphology of a novel cyanobacterium and characterization of light-harvesting complexes from it: implications for phycobiliprotein evolution. Proc Natl Acad Sci USA 78: 6888–6892 (1981).
27. Larkum AWD: Evolution of chlorophylls, light harvesting systems and photoreaction centres. In: Murata N (ed) Research in Photosynthesis, pp. 475–482. Kluwer Academic Publishers, Dordrecht, Netherlands (1992).
28. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, NY (1982).
29. Marco E, Martinez I, Ronem Tarazi M, Orus MI, Kaplan A: Inactivation of *ccmO* in *Synechococcus* sp. strain PCC 7942 results in a mutant requiring high levels of CO_2. Appl Environ Microbiol 60: 1018–1020 (1994).
30. Martin W, Somerville CC, Loiseaux-de Goër S: Molecular phylogenies of plastid origins and algal evolution. J Mol Evol 35: 385–404 (1992).
31. Morden CW, Delwiche CF, Kuhsel M, Palmer JD: Gene phylogenies and the endosymbiotic origin of plastids. BioSystems 28: 75–90 (1992).
32. Morden CW, Golden SS: Sequence analysis and phylogenetic reconstruction of the genes encoding the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase from the chlorphyll *b*-containing prokaryote *Prochlorothrix hollandica*. J Mol Evol 32: 379–395 (1991).
33. Morse D, Salois P, Markovic P, Woodland Hastings J: A nuclear-encoded form II RuBisCo in dinoflagellates. Science 268: 1622–1624 (1995).
34. Newman J, Mann NH, Carr NG: Organization and transcription of the class I phycoerythrin genes of the marine cyanobacterium *Synechococcus* sp. WH7803. Plant Mol Biol 24: 679–683 (1994).
35. Ogawa T, Amichay D, Gurevitz M: Isolation and characterization of the *ccmM* gene required by the cyanobacterium *Synechocystis* PCC6803 for inorganic carbon utilization. Photosyn Res 39: 183–190 (1994).
36. Ogawa T, Marco E, Orus MI: A gene (*ccmA* required for carboxysome formation in the cyanobacterium *Synechocystis* sp. strain PCC6803. J Bact 176: 2374–2378 (1994).
37. Palenik B, Haselkorn R: Multiple evolutionary origins of prochlorophytes, the chlorophyll *b*-containing prokaryotes. Nature 355: 265–267 (1992).
38. Parry MAJ, Madgwick P, Parmar S, Cornelius MJ, Keys AJ: Mutations in loop six of the large subunit of ribulose-1,5-bisphosphate carboxylase affect substrate specificity. Planta 197: 109–112 (1992).
39. Pichard SL, Frischer ME, Paul JH: Ribulose bisphosphate carboxylase gene expression in subtropical marine phytoplankton populations. Mar Ecol Prog Ser 101: 55–65 (1993).
40. Prézelin BB, Glover HE, Campbell L, 469-480. MB: Effects of light intensity and nutrient availability on diel patterns of cell metabolism and growth in populations of *Synechococcus* spp. Mar Biol 95: 469–480 (1987).
41. Price GD, Badger MR: Evidence for the role of carboxysomes in the cyanobacterial CO_2- concentrating mechanism. Can J Bot 69: 963–973 (1991).
42. Price GD, Howitt SM, Harrison K, Badger MR: Analysis of a genomic DNA region from the cyanobacterium *Synechococcus* sp. strain PCC7942 involved in carboxysome assembly and function. J Bact 175: 2871–2879 (1993).

43. Pridmore RD: New and versatile cloning vectors with kanamycin-resistance marker. Gene 56: 309–312 (1987).
44. Read BA, Tabita FR: High substrate specificity factor ribulose bisphosphate carboxylase/oxygenase from eukaryotic marine algae and properties of recombinant cyanobacterial rubisco containing 'algal' residue modifications. Arch Biochem Biophys 312: 210–218 (1994).
45. Rowan R, Whitney SM, Fowler A, Yellowlees D: RubisCO in marine symbiotic dinoflagellates: form II enzymes in eukaryotic oxygenic phototrophs encoded by a nuclear multigene family. Plant Cell 8: 539–553 (1996).
46. Saitou N, Nei M: The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425 (1987).
47. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).
48. Scanlan DJ, Bloye SA, Mann NH, Hodgson DA, Carr NG: Construction of *lacZ* promoter probe fusions for use in *Synechococcus*: application to the identification of CO_2-regulated promoters. Gene 90: 43–49 (1990).
49. Scanlan DJ, Mann NH, Carr NG: The response of the picoplanktonic marine cyanobacterium *Synechococcus* species WH7803 to phosphate starvation involves a protein homologous to the periplasmic phosphate-binding protein of *Escherichia coli*. Mol Microbiol 10: 181–191 (1993).
50. Shimada A, Kanai S, Maruyama T: Partial sequence of ribulose-1,5-bisphosphate carboxylase/oxygenase and the phylogeny of *Prochloron* and *Prochlorococcus* (Prochlorales). J Mol Evol 40: 671–677 (1995).
51. Sweeney BM, Borgese MB: A circadian rhythm in cell division in a prokaryote, the cyanobacterium *Synechococcus* WH7803. J Phycol 25: 183–186 (1989).
52. Tabita FR: The biochemistry and molecular regulation of carbon dioxide metabolism in cyanobacteria. In: Bryant DA (ed) The Molecular Biology of the Cyanobacteria, pp. 299–329. Kluwer Academic Publishers, Dordrecht, Netherlands (1994).
53. Tabita FR: The biochemistry and metabolic regulation of carbon metabolism and CO_2 fixation in purple bacteria. In: Blankenship RE, Madigan MT, Bauer CE (eds) Anoxygenic Photosynthetic Bacteria, pp. 885–914. Kluwer Academic Publishers, Dordrecht, Netherlands (1995).
54. Thompson JD, Higgins DG, Gibson TJ: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 22: 4673–4680 (1994).
55. Urbach E, Robertson DL, Chisholm SW: Multiple evolutionary origins of prochlorophytes within the cyanobacterial radiation. Nature 355: 267–270 (1992).
56. Valentin K, Zetsche K: The genes of both subunits of ribulose-1,5-bisphosphate carboxylase constitute an operon on the plastome of a red alga. Mol Gen Genet 16: 203–209 (1989).
57. Waterbury J, Watson S, Guillard RRL, Brand L: Widespread occurrence of a unicellular, marine, planktonic, cyanobacterium. Nature 277: 293–294 (1979).
58. Waterbury JB, Rippka R: Order Chroococcales. In: Kreig NR, Holt JB (eds) Bergey's Manual of Systematic Bacteriology, pp. 1728–1746. Williams and Wilkins, Baltimore (1989).
59. Whitney SM, Shaw DC, Yellowlees D: Evidence that some dinoflagellates contain a ribulose-1,5-bisphosphate carboxylase/oxygenase related to that of the alpha-proteobacteria. Proc R Soc London Ser B 259: 271–275 (1995).
60. Whitney SM, Yellowlees D: Preliminary investigations into the structure and activity of ribulose bisphosphate carboxylase from two photosynthetic dinoflagellates. J Phycol 31: 138–146 (1995).
61. Wilbanks SM, Glazer AN: Rod structure of a phycoerythrin II-containing phycobilisome. I. Organization and sequence of the gene cluster encoding the major phycobiliprotein rod components in the genome of marine *Synechococcus* sp. WH 8020. J Biol Chem 268: 1226–1235 (1993).
62. Wood AM, Townsend D: DNA polymorphism within the WH7803 serogroup of marine *Synechococcus* spp. (cyanobacteria). J Phycol 26: 567–585 (1990).