Phylogenetic diversity of *Synechococcus* in the Chesapeake Bay revealed by Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) large subunit gene (*rbcL*) sequences

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ABSTRACT: In order to understand how *Synechococcus* in the estuarine environment (Chesapeake Bay) are phylogenetically related to other known marine *Synechococcus*, partial *rbcL* gene sequences from 25 strains of *Synechococcus* spp. isolated from estuarine, coastal and oceanic waters were sequenced. The *rbcL* gene phylogeny showed that Chesapeake Bay *Synechococcus* isolates together with other marine *Synechococcus* spp. formed a monophyletic group which belongs to the form IA RuBisCO. All the Chesapeake Bay *Synechococcus* were able to grow in a wide range of salinity (0 to 30‰), and most of them belong to the Marine Cluster B (MC-B). Interestingly, several phycoerythrin (PE)-containing *Synechococcus* isolated from the bay were clustered in the MC-B group, which had previously only contained the non-PE *Synechococcus*. A set of PCR primers was developed to specifically amplify the *rbcL* gene from natural marine *Synechococcus* populations. After screening 232 clones randomly selected from 5 clone libraries (built on 5 estuarine samples respectively), at least 7 different *rbcL* genotypes or the operational taxonomic units (OTUs) were identified. Despite the great genetic diversity among the OTU sequences, they were all clustered with 13 Chesapeake Bay isolates. The distribution frequency of these OTUs varied dramatically from the upper to lower bay. Our results suggest that the Chesapeake estuary provides an ideal environment for the MC-B type *Synechococcus* populations to thrive. Marine *Synechococcus* appear to adapt well to various ecological niches, and a clear boundary solely based on phenotypic features may not exist when more and more novel ecotypes and genotypes are unveiled with molecular tools.

KEY WORDS: *Synechococcus* · Estuary · *rbcL* gene · Phylogeny · Classification

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INTRODUCTION

Phycobilisome-containing unicellular cyanobacteria of the genus *Synechococcus* are among the most abundant members of the picophytoplankton in the open ocean (Johnson & Sieburth 1979, Waterbury et al. 1979). Marine *Synechococcus* species contribute 5 to

30 % of primary production in the surface waters of world's oceans (Waterbury et al. 1979). The *Synechococcus* group (Chroococcales) is a provisional assemblage that was loosely defined as unicellular coccoid to rod-shaped cyanobacteria (<3 µm in diameter) whose cells divided by binary fission in a single plane (Waterbury & Rippka 1989). Based on morphological, physio-

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logical and chemical properties and DNA base ratios, *Synechococcus* spp. have been divided into 6 clusters: Cyanobacterium, *Synechococcus*, Cyanobium, Marine Clusters (MC) A, B, and C (Waterbury & Rippka 1989). It is now known that MC-A, MC-B, the Cyanobium cluster and the *Prochlorococcus* group formed a monophyletic group, distinct from freshwater *Synechococcus* and the MC-C cluster (Fuller et al. 2003). All strains in the MC-A cluster contain phycoerythrin (PE) as their major light-harvesting pigment. PE-containing *Synechococcus* strains can be distinguished by the presence or absence of phycourobilin (PUB) and the ratio of PUB to phycoerythrobilin (PEB) contained in the individual cells. All strains in MC-B and MC-C contain only phycocyanin as their major light harvesting pigment, but do not contain PE (Waterbury & Rippka 1989). Non-PE type is also called PC type in this study.

The PE type *Synechococcus* is a major primary producer in pelagic oceans (Campbell et al. 1983, Campbell & Iturriaga 1988, Olson et al. 1990), and the phylogenetic relationship among PE isolates has been extensively examined (Wood & Townsend 1990, Urbach et al. 1992, 1998, Toledo & Palenik 1997, Toledo et al. 1999, Rocap et al. 2002, Fuller et al. 2003). Divergence of PE strains based on the 16S rDNA sequences appeared to be narrow (<4%). The term 'microdiversity' has been used to describe a narrow genetic niche for unicellular cyanobacteria including *Synechococcus* and *Prochlorococcus* (Fuhrman & Campbell 1998, Moore et al. 1998). The RNA polymerase gene (*rpoC1*) has been used to study the phylogenetic linkage among the PE type *Synechococcus* (Toledo & Palenik 1997, Toledo et al. 1999). The *rpoC1* based phylogeny appeared to correlate well with the motility of PE *Synechococcus*. Most recently, the phylogenetic study based on the 16S–23S ribosomal DNA internal transcribed spacer (ITS) sequences divided the MC-A *Synechococcus* into 6 clades (Rocap et al. 2002).

In order to further resolve the phylogeny of the MC-B strains, more *Synechococcus* strains isolated from coastal estuaries are needed. Compared with other *Synechococcus* clusters, MC-B is not a well-characterized group because it only contains 4 strains (WH8007, WH8101, WH5701, and PCC7001) (Waterbury & Rippka 1989). Recently, WH8101 and PCC7001 have been re-classified into MC-A and the *Cyanobium* cluster, respectively (Herdman et al. 2001). Although marine *Synechococcus* and *Prochlorococcus* frequently co-exist in oceanic waters, the former dominates in coastal waters (Partensky et al. 1999). Chromatic adaptation by *Synechococcus* enables this group to adapt to broad ecological niches; a number of different genotypes or ecotypes of *Synechococcus*

have been identified in natural environments (Palenik 2001, Ferris et al. 2003). It is now known that *Synechococcus* are also abundant and dynamic members of the plankton in coastal estuaries. For examples, the abundance of *Synechococcus* in the Bedford Basin varied from a few cells to 4.5×10^5 cells ml⁻¹, and was greatly influenced by the temperature (Li 1998). The seasonal survey in the Chesapeake Bay showed that *Synechococcus* density reached the maximum of 1.3×10^6 cells ml⁻¹ in the summer and dropped to the minimum of 4.9×10^2 cells ml⁻¹ in the winter (K. Wang & F. Chen unpubl. data). In a recent review, (Scanlan & West 2002) indicated that *Synechococcus* from estuarine or coastal waters have not been well studied so far and are likely under-represented in *Synechococcus* culture collections and within phylogenetic trees as well. Is it possible that *Synechococcus* in coastal estuaries exhibit greater genetic diversity compared to the PE type, due to their complex habitats?

Ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) is a key enzyme in the primary pathway of photosynthetic carbon fixation of all primary producers. RuBisCO catalyzes the assimilation of CO₂ to organic carbon via the Calvin-Benson cycle. The enzyme is made of large and small subunits. The large subunit is responsible for carbon fixation (Miziorko & Lorimer 1983). This enzyme has been found to have 2 natural forms, Forms I and II. Form I contains 8 large and 8 small subunits (L₈S₈), encoded by *rbcL* and *rbcS*, respectively. Form II is comprised of large subunits only, usually as a dimer (L₂) (Watson & Tabita 1997). The Form I RuBisCOs are believed to have evolved in response to the decline of CO₂ and the emergence of oxygen while the global atmosphere changed (McFadden & Tabita 1974). According to the phylogeny of the *rbcL* gene, the Form I RuBisCOs can be divided into 4 different forms (A to D). Form IA consists of 2 marine cyanobacteria (marine *Synechococcus* WH7803 and *Prochlorococcus marinus*) and chemolithoautotrophs such as *Chromatium vinosum*, *Thiobacillus denitrificans*, and *T. ferrooxidans* (Watson & Tabita 1997). Form IB contains chloroplasts of terrestrial plants, green algae and cyanobacteria (e.g. *Anabaena*, freshwater *Synechococcus* and *Synechocystis*, *Prochloron*). Form IC has been found in a few alpha- and delta-proteobacteria, and many non-green algae carry Form ID RuBisCO (Watson & Tabita 1997). Unlike the ribosomal RNA genes which are universal for all living organisms, RuBisCO (the *rbcL* gene) is found primarily in photoautotrophs and chemolithotrophs. Because RuBisCO plays a central role in photosynthesis, the phylogenetic analyses based on the *rbcL* gene sequences have provided many new insights on the evolution of photosynthesis (Clegg 1993). The *rbcL* gene has been used as a phylogenetic marker to inves-

tigate the genetic diversity of marine phytoplankton communities (Pichard et al. 1997) and microorganisms living in the deep-sea (Elsaied & Naganuma 2001) and in groundwater (Alfreider et al. 2003).

In this study, our objective was to understand the phylogenetic diversity of *Synechococcus* living in the Chesapeake Bay, the largest and probably the most complex estuary in the US. The partial *rbcL* sequences from 13 Chesapeake Bay *Synechococcus* isolates and 12 Woods Hole *Synechococcus* isolates were sequenced and used for phylogenetic reconstruction. The *rbcL* gene phylogeny revealed that the vast majority of Chesapeake Bay *Synechococcus* isolates belong to the MC-B group. We also developed a new set of PCR primers specific to the *rbcL* gene of marine *Synechococcus* as a tool for exploring the genetic diversity of *Synechococcus* populations in the bay. The environmental *rbcL* sequences showed that *Synechococcus* living in the bay are diverse, but still closely related to Chesapeake Bay *Synechococcus* isolates.

MATERIALS AND METHODS

***Synechococcus* strains.** *Synechococcus* strains used in this study are described in Table 1. The Chesapeake Bay *Synechococcus* spp. (with CB prefix) were isolated in our laboratory. To isolate CB strains, water samples

collected from the bay were pre-incubated to increase *Synechococcus* density (Waterbury & Willey 1988). A pour plating method (Brahamsa 1996) was used to isolate *Synechococcus* strains from the pre-incubated waters. Marine *Synechococcus* strains were grown in the SN medium (Waterbury et al. 1986) at 25°C in constant light (20 to 30 $\mu\text{E m}^{-2} \text{s}^{-1}$) in an illuminated incubator. The salinity of SN media was adjusted to be close to the salinity of water samples. The motility was observed by phase-contrast microscopy using a wet mounts of cultures. Cultures of marine *Synechococcus* isolates WH7803, WH7805, WH8101 and WH8103 were provided by B. Binder at the University of Georgia and the other WH strains were from L. Campbell's laboratory. WH8007 and WH5701 were purchased from the Center for Culture of Marine Phytoplankton (CCMP) at Bigelow Laboratory (Maine, USA).

***In vivo* pigment absorption spectra.** *In vivo* pigment absorption spectra for the *Synechococcus* strains isolated from Chesapeake Bay and some Woods Hole *Synechococcus* isolates were determined between 400 to 800 nm in 1 nm intervals using a Beckman DU640 spectrophotometer. Cultures (2 ml) under exponential growth were used to measure the pigment absorption spectra of *Synechococcus*.

Enumeration of *Synechococcus* cells. To determine the *Synechococcus* abundance in a water sample, a 10 ml water sample was filtered onto a 0.2 μm pore size

Table 1. *Synechococcus* strains used in this study

Strain	PE	PUB:PEB	Motility	Location	Source	Accession no.
CB0101	–	no PUB	+	Inner Harbor, CB	This study	AY244815
CB0102	–	no PUB	–	Inner Harbor, CB	This study	AY244816
CB0103	–	no PUB	–	Inner Harbor, CB	This study	AY244817
CB0104	–	no PUB	–	Inner Harbor, CB	This study	AY244818
CB0201	–	no PUB	–	Stn 818, CB	This study	AY244819
CB0203	–	no PUB	–	Stn 818, CB	This study	AY452725
CB0204	–	no PUB	–	Stn 707, CB	This study	AY244820
CB0205	+	no PUB	+	Stn 818, CB	This study	AY244833
CB0206	+	no PUB	+	Stn 707, CB	This study	AY452726
CB0207	+	no PUB	+	Stn 707, CB	This study	AY452727
CB0208	+	no PUB	+	Stn 707, CB	This study	AY452728
CB0209	+	no PUB	–	Stn 707, CB	This study	AY452729
CB0210	+	no PUB	–	Stn 707, CB	This study	AY452730
WH5701	–	no PUB	–	Long Island Sound	Guillard R	AY244822
WH8007	–	no PUB	–	Gulf of Mexico	Provasoli L	AY244825
WH8101	–	no PUB	–	WHOI Harbor	Valois F	AF081831
WH8102	+	high	+	Sargasso Sea	Waterbury J	AF081832
WH8103	+	high	+	Sargasso Sea	Waterbury J	AY244827
WH8108	+	high	–	Sargasso Sea	Waterbury J	AY244831
WH7803	+	low	–	Sargasso Sea (25 m)	Waterbury J	U46156
WH8104	+	low	–	Sargasso Sea	Waterbury J	AY244828
WH8111	+	low	–	Sargasso Sea	Waterbury J	AY244832
WH7805	+	no PUB	–	Sargasso Sea	Brand L	AF081833
WH8006	+	no PUB	–	Gulf of Mexico	Waterbury J	AY244824
WH8008	+	no PUB	–	Gulf of Mexico	Waterbury J	AY244826

25 mm black polycarbonate membrane filter (Osmonics). A Nikon Eclipse E400 epifluorescence microscope was used to count the total *Synechococcus* cells, PC and PE type cells, respectively. The total cells were enumerated under green light excitation (528 to 553 nm). Under blue light excitation (465 to 495 nm), the PE cells showed bright yellow-orange color while the PC cells showed dim red in the same field. The PE cells were counted under the blue excitation. The difference between total counts and PE cell counts yielded the PC cell counts. At least 20 fields with the minimal of 200 cells per water sample were counted.

Oligonucleotide primers. When we started this project in 1998, there was only 1 complete *rbcl* gene sequence available for marine *Synechococcus* (WH7803) in the GenBank (Watson & Tabita 1996). Although PCR primers became available to amplify a 480 to 483 bp fragment of *rbcl* gene in 1997 (Pichard et al. 1997), the primers are degenerate and not specific for marine *Synechococcus*. A reverse degenerate PCR primer that is specific for Form IA *Synechococcus* has recently been published (Wawrik et al. 2003). Our goal was to design a set of non-degenerate primers specific for marine *Synechococcus* which can be extended for denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphisms (T-RFLP) analysis of natural marine *Synechococcus* populations.

In this study, 2 primer sets were used to amplify the *rbcl* gene from various marine *Synechococcus* isolates and a third primer set was developed for environmental *Synechococcus*. The first primer set (WHF1 and WHR1) was used to amplify the *rbcl* gene from all the PE strains. This primer set was designed based on aligned nucleotide sequences of the *rbcl* genes of all the freshwater cyanobacteria and WH7803. The WHF1/WHR1 are non-degenerate primers and specific for WH7803 strain. The sequences of WHF1 and WHR1 are 5'-ATGAGCAAGAAGTACGA-3' (sequence position of 1–18 as for WH7803) and 5'-GGTCTCCTGCTCGGACAG-3' (sequence position of 655–670 as for WH7803), respectively. The *rbcl* gene from all the PE strains in Table 1 was amplified with WHF1/WHR1, and sequenced afterwards.

The second set of *rbcl* primers (CF1 and CR1) was designed based on the conserved regions on the *rbcl* sequences of 10 known PE strains and other cyanobacterial strains. This primer set was used to amplify the *rbcl* genes from PC strains that could not be amplified with WHF1/WHR1. The sequences for CF1 and CR1 are 5'-AC(TC)TACT(GA)GACTCC(TC)GAT TAC-3' (sequence position of 43–63 as for WH7803) and 5'-GAA(GA)GGCTG(GA)GAGTT(GA)ATGTT-3' (sequence position of 589–610 as for WH7803). The *rbcl* gene from all the PC strains was amplified using CF1/CR1.

The third primer set MSF1 and MSR1 was designed to specifically amplify the *rbcl* gene from the Marine *Synechococcus* Clade (in Form IA, see Fig. 4). The primer MSF1 contained oligonucleotide sequence 5'-GGTCCACTGTGTGGTCCGAGG-3' which corresponds to the sequence position of 88–105 (173–192) as for WH7803. The primer MSR1 has the nucleotide sequence 5'-GTTCTCGTCGTCCTTGGTGAAGTC-3', which corresponds to the sequence position of 568–591 as for WH7803. The non-degenerate MSF1/MSR1 primers were tested against the natural bacterial communities in the Chesapeake Bay, with the goal to develop rapid fingerprinting techniques like DGGE for monitoring *Synechococcus* populations in the natural marine ecosystem.

Field sample collection. Water samples were collected from 2 m deep water of Chesapeake Bay and other estuarine stations (Fig. 1) using Niskin bottles on board the RV 'Cape Henlopen' on September 26–30, 2002. Water samples from the Roosevelt Inlet, Lewes, Delaware, were collected from surface water at the dock using a plastic bucket. For each station, 250 ml water was filtered through 0.2 µm pore-size polycarbonate filters (47 mm diameter, Millipore) and microbes retained on the filters were stored at –20°C until DNA extraction.

Nucleic acid extraction. Total DNA was extracted according to a protocol developed by Schmidt et al. (1991) with minor modifications. Briefly, DNA from the bacterial community was extracted by treating with lysozyme and proteinase K concomitant with phenol extraction and isopropanol precipitation. The DNA was dissolved in ddH₂O and stored at –20°C for further analysis.

PCR amplification. For *Synechococcus* isolates, DNA released from boiled cultures was used as templates. For preparation of cell lysis, *Synechococcus* cultures were harvested by centrifugation at 10000 rpm for 5 min. Culture pellets were re-suspended in 50 µl of lysis solution (0.1M Tris-HCl, pH 8.0, 0.01 % P-40) and boiled for 5 min. PCR was conducted in 0.2 ml tubes with a final volume of 25 µl reaction mixture, which contained 1 Unit of Taq DNA polymerase (Promega), 200 µM of each dNTP, 1.5 mM of MgCl₂, 1 µM each of oligonucleotide primer and 1 µl of cell lysates. For the primer set WHF1 and WHR1, the PCR amplification profile consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The PCR condition for the primer sets CF1 and CR1 was similar to WHF1/WHR1, except that the annealing temperature for CF1/CR1 was increased to 55°C.

The primer set MSF1/MSR1 was used to amplify the *rbcl* gene from the environmental DNAs. The PCR program for primers MSF1/MSR1 included an initial

Fig. 1. Sampling locations in the Chesapeake Bay. Water samples were taken at 2 m depth. At the time of sampling, the salinity at Stns 908, 818, 707, and Potomac River (PR) was 15, 17, 26, and 0‰, respectively. The salinity of Lewes Dock (38°46'N, 75°08'W) in the Delaware Bay (not shown on the map) is about 8‰

denaturation at 94°C for 5 min and 10 touchdown cycles of denaturation at 94°C for 1 min, annealing at 65°C (with temperature decreasing 1°C per cycle) for 1 min, and extension at 72°C for 1 min, followed by 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The final extension of PCR amplification was at 72°C for 10 min. PCR amplicons were examined by electrophoresis in 1% agarose gel stained with ethidium bromide in 0.5X TBE buffer. Gel images were captured and analyzed using Kodak EDAS 290 electrophoresis documentation and analysis system (Eastman Kodak).

Clone library construction. The 5 PCR products amplified from environmental samples were cloned. Each PCR product was purified by using Wizard PCR Prep DNA Purification System (Promega). The purified

DNA fragments were cloned into the pGEM-T Easy cloning vector (Promega) and then transformed into JM109 competent cells (Promega) according to the manufacturer's instructions. Positive clones (white-colonies) were picked randomly and transferred onto a new agar plate for further use.

Restriction fragment length polymorphism (RFLP) analysis. To avoid redundant sequencing, the *rbcl* clones amplified from 5 estuarine microbial communities were pre-screened with RFLP. About 50 to 60 positive clones from each clone library were randomly picked and the plasmid inserts were PCR-amplified with vector-specific primers T7 (5'-TAATACGACTCACTATAGGGCGA-3') and SP6 (5'-ATTTAGGTGACACTATAG-3') along with Taq DNA polymerase (Promega). PCR amplification cycles involved a 3 min initial denaturation at 94°C and followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, and a 5 min final extension at 72°C. Once a clone was confirmed to contain an insert of the appropriate size (ca. 510 bp), the insert was subjected to RFLP analysis. A subsample (10 µl) from each PCR mixture was digested with 5 U *Hae*III in 1× Buffer C (Promega) at 37°C overnight in a final reaction volume of 25 µl. Digested DNA was separated by 2% agarose gel electrophoresis as described above. The resulting RFLP patterns were examined and compared by visual check. The distinguishable RFLP patterns were assigned as operational taxonomic units (OTUs).

Sequencing and phylogenetic analysis. Prior to sequencing, PCR products were purified using the DNA Purification System, Wizard DNA Clean-up (Promega) according to the manufacturer's protocol. Purified PCR products were then sequenced using an automated sequencer ABI310 (PE Applied Biosystems) in the Biological and Analytical Laboratory at the Center of Marine Biotechnology, University of Maryland Biotechnology Institute. Both strands of the DNA were sequenced if there were uncertain nucleotides in the first strand of DNA. Sequence alignment and phylogenetic reconstruction were conducted using Mac Vector 7.1 program (GCG). Jukes-Cantor distance matrix analysis was used to calculate the distances from the aligned DNA sequences, and the neighbor-joining method was used to construct phylogenetic tree. To construct a phylogenetic tree based on the deduced amino acids, the pairwise similarity was calculated by using Blosom30 as matrix with open gap penalty of 10.0 and extend gap penalty of 0.1. The phylogenetic tree was constructed by using the neighbor-joining method based on 166 amino acid residues. Bootstrap values for both trees were obtained from analysis of 1000 re-samplings of the data set.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been

deposited in GenBank. The assigned accession numbers for the *Synechococcus* strains used in this study are shown in Table 1. The assigned accession numbers for OTUs are as follows: OTU1 (AY453140), OTU2 (AY453144), OTU3 (AY453145), OTU4 (AY453148), OTU5 (AY453149), OTU6 (AY453150), OTU7 (AY453152). The reference strains retrieved from GenBank included *Anabaena* sp. PCC7120 (P00879), *Chromatium vinosum* (M26396), *Cylindrotheca* sp. (P24673), *Heterosigma akashiwo* (M24288), *Nitrobacter winogradskyi* (AF109915), *Prochlorococcus marinus* CCMP1378 (ZP_00104542), *P. marinus* MIT9313 (ZP_00113526), *Prochlorothrix hollandica* (P27568), *Rhodobacter capsulatus* (L82000), *Synechococcus* PCC6301 (J01536), *Synechococcus* PCC7002 (Q44176), *Synechocystis* PCC6803 (D64000), *Thiobacillus denitrificans* (L42940), *T. ferrooxidans* A (Q07087), *T. ferrooxidans* B (P28895), and *Trichodesmium erythraeum* IMS101 (ZP_00072133).

RESULTS AND DISCUSSION

Characterization of Chesapeake Bay *Synechococcus* isolates

Among 13 CB strains, 7 strains are the PC type (or non-PE type) and 6 strains are the PE type (Table 1). The absorption spectra of Chesapeake Bay PC strains were similar to that of *Synechococcus* WH8101 in MC-B, while the PE strains isolated from the bay have an absorption spectrum similar to WH7805 (data not shown). Microscopic examination suggested that the vast majority of colonies consist of small unicellular cyanobacteria ranging in size from 1 to 3 μm . Through the course of isolation of *Synechococcus* from the Chesapeake Bay, we found that most of colonies (80 to 95% of all colonies) recovered from the Baltimore Inner Harbor and upper Chesapeake Bay (with salinity <15‰) were green strains. The percentage of the green colonies grown on the plate decreased to 56 to 65% at the mouth of the bay (Stn 707) where salinity is typically in the range of 20 to 30‰. The percentage of pigment types estimated based on the colonies recovered from the pre-incubated samples may not reflect the actual distribution of PC and PE *Synechococcus* in the bay. Further examination of original water samples using epifluorescence microscopy showed that PC type *Synechococcus* were predominant (ca. 87%) in the upper bay and Baltimore Inner Harbor, while PE type *Synechococcus* could account for approximately 82% of total *Synechococcus* populations in the lower bay (Stn 707) (Fig. 2). The ratio of PC versus PE type appeared to decrease from the upper to the lower bay (Fig. 2).

Six CB strains (CB0101, CB0104, CB0201, CB0205, CB0208, CB0210) were able to grow in the SN medium with wide range of salinity (0, 15, and 30‰, respectively), although their growth rate varied slightly with the amount of salt amended. In contrast, WH7803 and WH7805 could not survive in the SN medium with 0‰ salinity. These data suggest that the Chesapeake Bay *Synechococcus* have been adapted to the estuarine ecosystem.

Interestingly, several motile strains were found in the lower bay where PE strains were relatively more abundant compared to the upper bay. Five CB strains (CB0101, CB0205, CB0206, CB0207, and CB0208) were motile, and 4 of them were PE type (Table 1). The motile *Synechococcus* strains were thought to be more common in the oligotrophic waters, and they are not usually found in nutrient-rich coastal regions (Olson et al. 1990, Palenik 1994, Toledo et al. 1999).

Most of Chesapeake Bay *Synechococcus* isolates belong to the MC-B cluster

The phylogenetic tree based on the *rbcl* DNA sequences of CB strains and the bay samples revealed that *Synechococcus* living in the Chesapeake Bay are very diverse (Fig. 3). The CB strains alone exhibited the genetic diversity equivalent to the Woods Hole PC strains WH5701, WH8007 and WH8101 which were isolated from Long Island Sound, the Pier Harbor of Woods Hole Oceanography Institute, and the Gulf of Mexico, respectively. All the PE strains (Woods Hole collection) isolated from the Sargasso Sea and the Gulf of Mexico by Waterbury and Brand (see Table 1) were clustered within the MC-A group. Surprisingly, all the Chesapeake Bay PE strains (except for CB0205) were not clustered with the Woods Hole PE isolates in the MC-A group (Fig. 3). Instead, the 5 PE strains (CB0206,

Fig. 2. Concentration of PC and PE type *Synechococcus* at Stns 908, 818 and 707, and in the Potomac River. Error bars represent SD of total cell counts. Closed bar: PE type; open bar: PC type

Fig. 3. Neighbor-joining tree of marine *Synechococcus* strains constructed using the partial *rbcL* nucleotide sequences (414 bases). OTUs included in this tree represent the 7 distinct environmental *rbcL* genotypes (shown in Fig. 6). Percentages in the parenthesis are the relative distribution frequency of each OTU among 233 *rbcL* clones analyzed. Numbers at tree branches indicate bootstrap values with 1000 replicates. Bootstrap values less than 50 are not shown. Scale bar indicates 0.02 substitution per site

CB0207, CB0208, CB0209 and CB0210) from the bay were closely related to WH8007, a member of MC-B (Fig. 3). Our data here suggested that the MC-B cluster not only contains the PC type *Synechococcus*, but also the PE type *Synechococcus*. Out of 13 CB isolates, 9 were grouped with WH8007, but none of them were closely related to WH5701. CB0104 appeared to be in between WH5701 and WH8007. We believe that at least 10 CB strains can be considered as belonging to

MC-B (or Subcluster 5.2) which currently only contains WH8007 and WH5701.

According to the *rbcL* phylogeny, WH8101 could be clustered within the MC-A group, but with a relatively deep and independent branch. Recently, WH8101 has been re-classified into the MC-A or subcluster 5.1 (Herdman et al. 2001), and the close relationship between WH8101 and the MC-A group were also supported by the 16S–23S ribosomal DNA internal transcribed sequences (Rocap et al. 2002) and 16S rDNA gene sequences (Fuller et al. 2003), respectively. With PE strains found in the MC-B cluster and PC strains in the MC-A cluster, it is believed that pigment types (PE or PC) are no longer a critical feature for separating MC-A and MC-B *Synechococcus*. Moreover, the designation of MC-B should be redefined when more data are available.

The *rbcL* gene phylogeny also demonstrated that motile strains are closely related, but not necessary monophyletic. For example, 4 motile CB strains (CB0101, CB0206, CB0207 and CB0208) were closely related in MC-B, while 1 motile CB strain (CB0205) was tightly associated with WH8102 and WH8103 in MC-A (Fig. 3). A previous study focused on the MC-A strains showed that motile strains of *Synechococcus* form a monophyletic group (Toledo et al. 1999). Our result and a recent study based on 16S rDNA gene sequences (Fuller et al. 2003) both indicated that motile strains could also be found in MC-B representing a second group of motile *Synechococcus*. These findings suggested that classification or clustering of *Synechococcus* should not be simply concluded based on certain phenotypic features like motility. Our effort for isolating bacterial strains from natural environments is limited. In reality, we may not be able to find a clear boundary among *Synechococcus* based on phenotypic characteristics when more and more ‘in-between’ strains are discovered. It seems very likely that the genus *Synechococcus* (Cyanobacteria) is polyphyletic and its classification needs to be re-evaluated (Honda et al. 1999, Robertson et al. 2001).

***Synechococcus* strains from estuarine, coastal and oceanic waters form a monophyletic group**

The phylogenetic affiliation at the amino acid sequence level showed that all the estuarine, coastal and oceanic *Synechococcus* used in this study, together with marine *Prochlorococcus marinus*, share a common ancestry and belong to the Form IA RuBisCO (Fig. 4). *Prochlorococcus marinus* MIT9313 and CCMP1378 were closely related to each other, and also formed a close relationship with the marine *Synechococcus* cluster. The chemoautotrophic bacteria (e.g.

Fig. 4. Phylogenetic tree constructed using the neighbor-joining method based on the aligned *rbcL* amino acid sequences (138 residues). Forms IA and IB are the 2 major groups of RuBisCO. Numbers at tree branches indicate bootstrap values with 1000 replicates. Bootstrap values less than 50 are not shown. Scale bar indicates 0.05 substitution per site

Chromatium vinosum, *Nitrobacter winogradskyi*, *Rhodobacter capsulatus*, *Thiobacillus denitrificans*, *T. ferrooxidans*) were in another branch of the Form IA RuBisCO. All the freshwater cyanobacteria (e.g. *Anabaena* PCC7120, *Synechococcus* PCC6301 and PCC7002 and *Synechocystis* PCC6803) and marine filamentous cyanobacteria *Trichodesmium erythraeum* were within the Form IB RuBisCO.

Despite the wide range of salinity distribution in the Chesapeake Bay, all the CB strains (13 isolates) are still more closely related to the WH strains than to the freshwater *Synechococcus*, which were grouped within the Form IB RuBisCO (Fig. 4). The CB and WH

Synechococcus strains formed a monophyletic group (termed marine *Synechococcus* Clade) that is closely related to marine *Prochlorococcus*. The close relationship between marine *Synechococcus* and marine *Prochlorococcus* revealed by the *rbcL* gene phylogeny is consistent with the previous studies based on the 16S rDNA sequences (Palenik & Haselkorn 1992, Urbach et al. 1992). The *rbcL* phylogeny also showed a close relationship between marine unicellular cyanobacteria and chemolithotrophic bacteria (Fig. 4). Our results further support previous studies in which the *Prochlorococcus marinus* and *Synechococcus* strain WH7803 *rbcL* sequences are found most closely related to the gamma-purple bacterial sequences (Watson & Tabita 1996, 1997). It is very likely that marine *Synechococcus* and *Prochlorococcus* acquired the *rbcL* gene from a chemolithotroph through lateral gene transfer (Watson & Tabita 1997).

Our result does not support a previous study in which marine *Synechococcus* WH8007 was clustered into the Form IB group based on the *rbcL* gene phylogeny (Picard et al. 1997). In our study, the *rbcL* gene sequence of WH8007, together with other marine *Synechococcus* were all clustered into the Form IA group. Picard et al. (1997) also indicated that the 2 *Prochlorococcus* strains were clustered together with freshwater *Synechococcus* in the Form IB group. However, the phylogenetic analysis based on the various molecular markers (e.g. 16S rDNA, 16S-23S ITS, *rpoC1* and *rbcL*) did not support such a close relationship between *Prochlorococcus* and freshwater *Synechococcus* (Palenik &

Haselkorn 1992, Urbach et al. 1992, Shimada et al. 1995, Watson & Tabita 1997, Badger et al. 2002, Rocap et al. 2002).

Development of marine *Synechococcus*-specific primers based on the *rbcL* gene

Because all marine *Synechococcus* form a unique cluster, a set of non-degenerate PCR primers that are specific to this group of unicellular cyanobacteria was developed. The upstream primer MSF1 is specific for marine *Synechococcus* strains while the downstream primer

MSR1 is specific for marine *Synechococcus*, marine *Prochlorococcus*, some chemoautotrophic bacteria and freshwater *Synechococcus*. The primer set (MSF1/MSR1) was tested against the bacterial DNA recovered from several estuarine samples where the salinity ranged from 0 to 30‰. In all cases, specific PCR amplification was achieved (Fig. 5), suggesting that this primer set is suitable to amplify the *rbcL* gene from *Synechococcus* populations in various marine environments.

There are several reasons for developing non-degenerate *rbcL* primers that are specific for marine *Synechococcus*. First, this primer set will allow us to explore the genetic diversity of *Synechococcus* in the natural marine environment. Non-degenerate primers can also be coupled with rapid fingerprinting techniques such as DGGE or T-RFLP to monitor the *Synechococcus* populations over temporal and spatial scales. Second, non-specific PCR amplification is greatly minimized using the *rbcL* primers because the *rbcL* gene mainly exists in phototrophs and some chemolithotrophs. PCR amplification with the *rbcL*

Fig. 5. PCR amplification of the *rbcL* gene from natural marine bacterial communities using the primer set MSF1/MSR1, which is specific for marine *Synechococcus*. Lanes 1 and 7 correspond to Molecular Weight Marker VI (Roche) (from top to bottom: 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154 bp, respectively). Lanes 2 to 6 correspond to bacterial communities from Stns 908 (Upper Bay), 818 (Mid Bay) and 707 (Lower Bay) of Chesapeake Bay, Potomac River, and Lewes Dock (Roosevelt Inlet, Delaware)

Fig. 6. Seven different *rbcL* genotypes or OTUs revealed by RFLP analysis (Lanes 2 to 8) of *rbcL* clone libraries (a total of 232 clones). Lanes 1 and 9 are Molecular Marker VI. The reversed image is shown

primers can improve the specificity and avoid the contamination problem associated with 16S rDNA primers. Third, the *rbcL* gene sequences (also *rpoC1* and ITS sequences) provide greater level of genetic resolution relative to the 16S rDNA gene sequences, and are particularly suitable for studying the phylogenetic relationship of closely related strains like *Synechococcus*. Finally, this primer set will allow us to study the ecological interaction between *Synechococcus* and cyanophage in the natural marine environment. Viruses that infect marine *Synechococcus* (synchophages) are ubiquitous and abundant in various marine environments (Suttle & Chan 1993, 1994, Waterbury & Valois 1993, Wilson et al. 1993, Lu et al. 2001). The group-specific PCR primers have been applied to investigate the genetic diversity of synchophages in the Chesapeake Bay and other marine environments (Fuller et al. 1998, Zhong et al. 2002, Marston & Sallee 2003, Wang & Chen 2004), so it would be ideal to have a specific primer set for their corresponding hosts. In order to understand the complex interaction between marine viruses and their microbial hosts, it is necessary to look at the genetic diversity of both viral and host communities (Fuhrman 1999).

Highly diverse *Synechococcus* in the Chesapeake Bay

At least 7 genotypes (or OTUs) of *Synechococcus* were found in the Chesapeake Bay based on the RFLP analysis of environmental *rbcL* clones (Fig. 6). All the estuarine stations except for Stn 707 contained at least 6 different OTUs (Fig. 7). The 7 OTUs recovered from the 4 stations in the Chesapeake Bay and Lewes Dock in the Delaware Bay were clustered within the marine *Synechococcus* clade (Fig. 3). Among 232 clones tested, 10% (OTUs 4 and 7) were clustered with PE

Fig. 7. Relative percentages of each OTU presented in each sampling location: Chesapeake Bay Stns 908, 818 and 707, Potomac River (PR) and Lewes Dock (LD). The relative distribution of each OTU among the total 232 clones is shown as 'Total'

strains in MC-A, 22% (OTUs 5 and 6) were related to WH8101 (PC strain in MC-A), and 68% (OTUs 1, 2 and 3) were affiliated with the MC-B group (Fig. 7). The composition of environmental *rbcl* sequences suggests that Chesapeake Bay is dominated by the MC-B type *Synechococcus*.

In the Chesapeake Bay, the distribution of these *Synechococcus* genotypes changed from the upper to lower bay (Fig. 7). For example, from the upper bay (Stn 908) to lower bay (Stn 707), OTU2 gradually decreased from 51 to 15%, while OTU3 steadily increased from 16 to 49%, respectively. The dramatic change of *Synechococcus* populations in the Chesapeake Bay is reflected by the complicated ecological habitats in the bay. The Chesapeake Bay is an incredibly complex ecosystem in which salinity, temperature and nutrients are very dynamic both temporally and spatially. High salinity seawater from the Atlantic Ocean enters the mouth of the bay, and gradually decreases towards north. Salinity levels in the bay could range from near 0 to 30‰ across the north–south transaction. In our study, the OTUs were selected based on the RFLP digestion patterns of one restriction enzyme, and actual genetic diversity of *Synechococcus* in the bay could be underestimated using this method. In the California Current, 7 genetically distinct groups of *Synechococcus* have been identified based on the *rpoC1* sequence data (Toledo & Palenik 1997, Toledo et al. 1999, Ferris & Palenik 1998, Palenik 2001).

CONCLUSION

Despite the great genetic diversity revealed by the *rbcl* gene sequences of *Synechococcus* isolates and environmental clones, *Synechococcus* populations in

the Chesapeake Bay appear to be dominated by the MC-B (or Subcluster 5.2) type *Synechococcus*. Several novel PE strains within the MC-B cluster were found in the lower bay. All the CB strains are halotolerant, suggesting they have been adapted to the Chesapeake estuary where salinity could change from almost 0 to 30‰. *Synechococcus* in estuarine, coastal and oceanic waters (at least for MC-A and MC-B) are monophyletic rather than widely dispersed across the *rbcl* phylogenetic tree. With the marine *Synechococcus*-specific primers available, it is now possible to explore the genetic diversity of marine *Synechococcus* populations. At least 7 different genotypes of *Synechococcus* from the bay have been identified, and in most cases 6 distinct genotypes could be found in one sample. Our long-term goal is to explore the co-variation of both *Synechococcus* and their phage simultaneously in the natural aquatic environments. The spatial and temporal variations of *Synechococcus* populations, together with synechophage populations in the Chesapeake Bay are currently under investigation.

Acknowledgements. We thank the crew of the RV 'Cape Henlopen'. We thank all the scientists who participated in the MOVE (Microbial Observatories of Virioplankton Ecology) led by E. Wommack, F. Chen and W. Coats. We also thank B. Binder for providing several WH *Synechococcus* strains. This work was supported by grants from the National Science Foundation (OCE-0049098, MCB-0132070 and MCB-0238515).

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*Editorial responsibility: Jed Fuhrman,
Los Angeles, USA*

*Submitted: July 24, 2003; Accepted: April 15, 2004
Proofs received from author(s): July 19, 2004*