Distinct Patterns of Picocyanobacterial Communities in Winter and Summer in the Chesapeake Bay[†]

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Received 27 November 2009/Accepted 25 February 2010

In the Chesapeake Bay, picocyanobacteria were usually 100-fold less abundant in winter than in summer. However, little is known about how picocyanobacterial populations shift between winter and summer in the bay. This is due mainly to undetectable winter picocyanobacterial populations in bacterial 16S rRNA clone libraries. In this study, the winter and summer picocyanobacterial populations in the bay were detected using picocyanobacterium-specific primers and were compared based on the analysis of rRNA internal transcribed spacer sequences. Temperature was found to be the dominant environmental factor controlling picocyanobacterial populations in the Chesapeake Bay. In the summer, marine cluster B *Synechococcus* dominated the upper bay, while a unique cluster, CB1 (marine cluster A [MC-A] *Synechococcus*), made up the vast majority in the middle and lower bay. In the winter, the picocyanobacteria shifted to completely different populations. Subclades CB6 and CB7, which belong to MC-A *Synechococcus* and *Cyanobium*, respectively, made up the entire winter picocyanobacterial populations in the bay. Interestingly, the winter members in subclade CB6 clustered closely with *Synechococcus* CC9311, a coastal strain known to have a greater capacity to sense and respond to changing environments than oceanic strains.

Unicellular cyanobacteria, or picocyanobacteria, are abundant and dynamic in the aquatic environment. They contribute to a significant portion of the total primary production in marine and freshwater environments (25, 28). Diverse picocyanobacteria that have adapted to unique environments are found in various aquatic environments (i.e., lakes, brackish water, coastal and open oceans) (1–5, 8, 13, 14, 18–20). Picocyanobacteria are usually more abundant in the warm season than in the cold season, especially in the temperate zone. Nearly 3 orders of magnitude of decline in picocyanobacterial cell density from summer to winter is noted in both temperate marine and freshwater ecosystems (17, 29). Temperature ranges may be a key environmental parameter dictating the distribution of picocyanobacteria, especially in temperate aquatic ecosystems. For example, temperature ranges for clades I and IV are very similar and are clearly below those of other *Synechococcus* clades (31). However, little is known about the genetic diversity of picocyanobacteria during the cold winter season, especially in temperate ecosystems.

The Chesapeake Bay is a large temperate aquatic ecosystem. The surface water can reach temperatures as high as 28 to 30°C in the summer and can drop to nearly freezing temperatures in the winter (11). The abundance of picocyanobacteria in the bay varies dramatically from summer to winter. The density of

picocyanobacteria (dominated by marine *Synechococcus*) in the bay reaches more than 10⁵ cells ml^{−1} in the summer but drops to a few hundred cells per milliliter in the winter (4, 12). Although many picocyanobacteria have been isolated from the Chesapeake Bay, none of them were isolated during the winter (3). Due to the low abundance of picocyanobacteria (less than 0.1% of the total prokaryotic community) in the winter, no picocyanobacterial sequences are recovered in the winter clone libraries of the Chesapeake Bay bacterioplankton communities (4, 12). Our current understanding of the composition of the picocyanobacterial population in the bay is based mainly on a limited analysis of warm-weather bacterial clone libraries. We still do not understand how picocyanobacteria in the bay survive the winter. It would be interesting to know whether a subset of the summer picocyanobacterial community can maintain low cell numbers through the winter or whether different *Synechococcus* populations are present in the winter.

In this study, the genetic diversity of winter and summer picocyanobacterial communities in the Chesapeake Bay was investigated using a newly designed PCR primer set that targets picocyanobacterium-specific sequences of rRNA operons. Interestingly, none of the picocyanobacterial sequences recovered in the winter were detected in the summer, suggesting that there is a dramatic shift in major picocyanobacterial populations from the warm to the cold season in the Chesapeake Bay.

MATERIALS AND METHODS

Water sample collection. Surface water samples (at a depth of 2 m) were collected aboard the research vessel *Cape Henlopen* at three stations along the longitudinal axis of the Chesapeake Bay on 7 to 11 February and 11 to 17 July 2005. Stations 858, 804, and 707 were chosen to represent the northern, middle, and southern areas of the bay, which covered a wide salinity gradient (Fig. 1; see also Table 1). At each station, a 500-ml subsample was taken from a 10-liter Niskin bottle and was filtered immediately through a 0.2-μm-pore-size polycar-

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[†] Supplemental material for this article may be found at <http://aem.asm.org/>.

[‡] Published ahead of print on 12 March 2010.

FIG. 1. Map of the Chesapeake Bay showing sampling stations (modified from reference 23 with permission of the publisher).

bonate filter (diameter, 47 mm; Millipore, Billerica, MA). The filters were stored at -20°C prior to DNA extraction. Water temperature and salinity were recorded with a Seabird 911 CTD (conductivity, temperature, and depth) instrument. W. Coats of the Smithsonian Environmental Research Center kindly provided chlorophyll *a* (Chl *a*) data. Viral, bacterial, and cyanobacterial abundances were measured based on protocols described elsewhere (11). Briefly, water samples fixed in 1% glutaraldehyde were filtered, and microbes were counted using a Zeiss Axioplan (Zeiss, Germany) epifluorescence microscope. Nutrients were analyzed in the Chemical Laboratory at the Horn Point Laboratory, University of Maryland Center for Environmental Science (UMCES).

DNA extraction and PCR amplification. Bacterial genomic DNA was extracted using a phenol-chloroform protocol as previously described (11). PCR primers specific for picocyanobacteria were designed based on the conserved regions of the 16S rRNA-internal transcribed spacer (ITS)-23S rRNA operon. PCR primers Picocya16S-F (TGGATCACCTCCTAACAGGG) and Picocya23S-R (CCTTC ATCGCTCTGTGTGCC) were designed based on 51 available sequences of

picocyanobacterial strains. The primers perfectly match other picocyanobacterial 16S and 23S rRNA gene sequences, as judged using the Probe Match function in the Ribosomal Database Project (<http://rdp.cme.msu.edu/>) and BLASTN, version 2.2.18 (30). This primer set was designed based on existing knowledge of picocyanobacterial diversity in the Chesapeake Bay (4). The Chesapeake estuary contains diverse groups of picocyanobacteria, including different subgroups of *Cyanobium* and clusters 5.1 and 5.2 of marine *Synechococcus*. PCR products were obtained using a touchdown PCR program (with gradually decreasing annealing temperatures). Thermal cycle denaturation was set for 30 s at 94°C , annealing for 50 s, and primer extension for 1 min at 72°C . The cycling program was followed by a 7-min primer extension step and a 4°C soak step. The annealing stage was set for $0.5^{\circ}\text{C}/\text{cycle}$, beginning at 58°C with increments to 54°C (8 total cycles), followed by 20 additional cycles at 55°C .

Cloning, sequencing, and phylogenetic analysis. PCR products recovered from the six samples described above were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A total of 145 clones from the six libraries were picked for sequence analysis. Clones were sequenced using BigDye Terminator chemistry and an ABI 3100 genetic analyzer (Applied Biosystems) at the Center of Marine Biotechnology, University of Maryland Biotechnology Institute (UMBI). All sequences obtained were carefully checked for chimeric artifacts using the BLAST (BLASTN) program (<http://www.ncbi.nlm.nih.gov/BLAST>), and chimeric sequences were excluded from the phylogenetic analysis. The rarefaction curves produced by DOTUR (21) were monitored to ensure that sufficient numbers of clones were sequenced for each clone library. Phylogenetic analyses were performed with ARB (www.arb-home.de) and the PHYLIP software package, version 3.68 (<http://evolution.genetics.washington.edu/phyliip/>) (7). Phylogenetic trees were constructed using a variety of methods, including neighbor joining (ARB), parsimony (PHYLIP), and maximum-likelihood DNAML (PHYLIP). Statistical evaluation of tree topologies was performed using bootstrap analysis with 1,000 replicates for neighbor joining and parsimony and 100 replicates for maximum-likelihood trees. After comparison of trees generated by different methods, a consensus tree was constructed by introducing multifurcations where the topology was not resolved.

Statistical analyses. S-LIBSHUFF, a tool in mothur version 1.7.2 (<http://www.mothur.org>) (22), implementing the Cramer-von Mises test statistic, was used to compare clone libraries and to determine the degree of similarity between them. Multivariate statistics was used to investigate the relation between bacterial community composition and explanatory variables. The software package CANOCO 4.5 for Windows (26) was used for all analyses. Sequences with similarities greater than 99% were considered to belong to the same phylotype and were defined as operational taxonomic units (OTUs) (21, 24). The data matrices containing the absence or presence of different OTUs at a 99% similarity cutoff or explanatory variables were $\log(x + 1)$ transformed before analysis. Initial detrended correspondence analysis suggested a linear character of the data response to the sample origin; therefore, canonical correspondence analysis (CCA) was used. The environmental factors best describing the most influential gradients were identified by manual forward selection. We used a Monte Carlo permutation test based on 499 random permutations to test the significance of the relationship between explanatory variables and community composition (P , <0.05).

Nucleotide sequence accession numbers. The ITS sequences obtained in this study have been deposited in GenBank under accession no. FJ547142 to FJ547238.

RESULTS AND DISCUSSION

The hydrological and biological features of winter and summer samples were quite different (Table 1). The average surface water temperature (from the three stations) was below 3°C in February and higher than 26°C in July. Salinity increased gradually from the upper to the lower bay in both seasons (Table 1). In the middle bay (station [Stn] 804), the salinity was lower in February (9.6 ppt) than in July (13.0 ppt), suggesting a stronger freshwater influence in the winter. The monthly river discharge into the bay supports the impact of freshwater (see Fig. S3 in the supplemental material). Overall, the river discharge in the winter was much greater than that in the summer. The total counts of picocyanobacteria in the summer were 100-fold higher than those in the winter (Table 1).

TABLE 1. Physical, chemical, and biological measurements of water samples collected in the upper, middle, and lower Chesapeake Bay^a in 2005

Measurement	Value for:					
	February 2005			July 2005		
	Stn 858	Stn 804	Stn 707	Stn 858	Stn 804	Stn 707
Water temp (°C)	3.1	2.7	2.9	26.8	27.3	23.9
Salinity (ppt)	7.2	9.6	18.0	8.1	13.0	19.1
Ammonium (μM)	3.5	1.1	1.9	7.6	1.7	1.85
Nitrate + nitrite (μM)	43.6	35.2	9.1	2.3	0.3	0.2
Phosphate (μM)	0.42	0.22	0.19	0.50	0.47	0.39
Picocyanobacterial counts (10 ³ cells ml ⁻¹)	0.89	9.6	2.7	96.3	798	484
Chlorophyll <i>a</i> concn (μg liter ⁻¹)						
Total	11.08	4.98	5.53	12.39	4.95	8.19
<3-μm-pore-size filter	0.70	0.39	0.12	2.28	3.95	4.04
Virus-like particles (10 ⁶ ml ⁻¹)	1.98	7.19	8.71	8.49	10.3	10.2
Bacterial counts (10 ⁶ cells ml ⁻¹)	0.74	1.23	1.23	4.53	9.72	9.55

^a Stn 858 is in the upper part of the Bay; Stn 804 is in the middle; and Stn 707 is in the lower part of the Bay.

Picophytoplankton (<3 μm) made up 50 to 80% of the total phytoplankton biomass in the middle and lower bay in July 2005, yet they contributed only a few percentiles to the total phytoplankton biomass in February 2005. During the winter, the total bacterial community contained less than 1% picocyanobacteria at all three stations.

Three new subclades of picocyanobacteria in the bay. The Chesapeake Bay contains unique and diverse picocyanobacteria. Five *Synechococcus* subclades (CB1, CB2, CB3, CB4, and CB5) consisted only of ITS sequences from the Chesapeake Bay (except for WH8007 in subclade CB5) (Fig. 2A). Subclade CB1, a unique summer picocyanobacterial population, contained 41 clones recovered from the lower and middle bay in July 2005 (Fig. 2A). Members of subclade CB1 (marine cluster A [MC-A] *Synechococcus*) dominated the middle and lower bay in the summer and may represent a special group of *Synechococcus* adapted to the medium-high salinity (20 to 28 ppt) in midsummer. All 59 winter clones across the bay clustered into two subclades, CB6 (winter I) and CB7 (winter II) (Fig. 2A). The clones within each winter subclade were closely related (Fig. 2B and C). Twenty clones in CB6 (winter I) were closely related to *Synechococcus* isolates CC9311, WH8020, and WH9908 (Fig. 2B), while 39 clones in CB7 (winter II) were closely related to many ITS sequences belonging to *Cyanobium* that were retrieved from brackish water in the Baltic Sea (Fig. 2C).

The winter Chesapeake Bay population was composed of different groups of picocyanobacteria than the summer Chesapeake Bay population. The close relationship between subclade CB6 and coastal strains such as CC9311 and WH8020 suggested that CB6 may represent the winter picocyanobacteria derived from the coastal water. On the other hand, the kinship between CB7 and brackish-water clones (from the Baltic Sea) (10) indicates that the members of CB7 may represent the winter picocyanobacteria living in the low-salinity end of the Chesapeake Bay (the upper bay). CB7 (winter II, freshwater-type) clones were more abundant than CB6 (winter I, seawater-type) clones during the winter (Table 2), and such a distribution pattern could be related to stronger freshwater

discharge during the winter (see Fig. S3 in the supplemental material). The greater influence of freshwater also resulted in relatively lower salinity at Stn 804 (middle bay) in the winter than in the summer (Table 1).

Population shift between winter and summer. The relationship between CB subclades and environmental variables was investigated using CCA, which resulted in three clusters separated by temperature, salinity, concentrations of ammonium, nitrate, and nitrite, and the ratio of N to P (Fig. 3). The first cluster represented the bay in the winter, with low temperatures and high nitrate and nitrite concentrations; the second cluster represented the middle and lower bay in the summer, with high temperatures and salinities; the third cluster represented the upper bay in the summer, with high temperatures and ammonium concentrations. The CCA suggests that the patterns observed were separated mainly by temperature (Fig. 3), based on manual forward selection of the best minimum set of explanatory variables.

The different winter and summer picocyanobacterial populations were also evident by statistical analysis of LIBSHUFF data (see Fig. S2 in the supplemental material), with a *P* value of <0.001. A total of 135 ITS clones were sequenced from the six clone libraries and were used for the phylogenetic analysis (Table 2). The rarefaction curves at a 98% similarity cutoff for the winter samples were saturated quickly due to their low sequence diversity (see Fig. S1 in the supplemental material). The genetic diversity of summer samples appeared to be higher than that of winter samples. Therefore, relatively more clones were sequenced from the summer samples than from the winter samples. Even with more clones being sequenced, the rarefaction curves for the summer samples still were not saturated, suggesting that the genetic diversity of summer *Synechococcus* is greater than we showed here. Despite the fact that only the major picocyanobacterial populations were detected in this study, none of the winter populations (CB6 and CB7), or very few, were detected in the summer. Since only two sample times were analyzed, it is possible that winter-related strains may persist during other months of the year. None of the winter clones were affiliated with marine cluster B (MC-B)

FIG. 2. (A) Phylogenetic relationships of ITS sequences. (B and C) Higher-resolution views of subclades CB6 (B) and CB7 (C). The TH group designations follow those of Haverkamp et al. (10) for sequences from the Baltic Sea. A consensus tree was constructed with bootstrap values of >90% (●) or 50 to 90% (○). Bootstrap values of <50% were omitted. The tree was rooted with *Synechococcus elongatus* PCC6301. Bar, 2% nucleotide sequence divergence. Clone designations consist of the number of the station from which the sample was obtained, the sampling time in parentheses ("705" stands for July 2005, and "205" stands for February 2005), and the number in the clone library. Clones from samples collected in July 2005 are shown in red, and clones from samples collected in February 2005 are shown in blue.

TABLE 2. Phylogenetic clades obtained from clone libraries and percentages of sequences in each clade

Phylogenetic clade	No. of clones (% of sequences)					
	February 2005			July 2005		
	Stn 858	Stn 804	Stn 707	Stn 858	Stn 804	Stn 707
<i>Synechococcus</i>						
CB1					19 (67.9)	22 (75.9)
CB3						2 (6.9)
CB4				5 (26.3)	4 (14.3)	3 (10.3)
CB5				14 (73.7)	5 (17.8)	2 (6.9)
CB6 (winter I)	6 (46.1)	6 (27.3)	8 (44.4)			
CB7 (winter II)	13 (53.9)	16 (72.7)	10 (55.6)			

Synechococcus, suggesting that MC-B members may not tolerate cold conditions. This is consistent with the fact that all the MC-B strains were isolated during the warm seasons (3). Efforts should be made, therefore, to isolate picocyanobacteria from water samples collected during the winter period.

The spatial distribution of summer picocyanobacterial populations was less uniform than that of the winter populations (Fig. 3). According to the CCA, station 858 was quite different in the summer from stations 804 and 707. CB5 dominated the upper bay, which was characterized by a higher ammonium concentration and lower salinity. Sequences within this subclade grouped within MC-B, i.e., typically brackish-water *Synechococcus* sequences. In contrast, sequences in CB1 originated from waters with higher

salinity and grouped within MC-A, the obligately marine *Synechococcus* group (Table 2).

Cold adaptation of picocyanobacteria in the Chesapeake Bay. Despite the low abundance of picocyanobacteria in the Chesapeake Bay in the winter, the picocyanobacterium-specific PCR primers allowed us to detect the “rare populations” and reveal the presence of different groups of picocyanobacteria in winter. The Chesapeake Bay winter populations in subclade CB6 were closely related to several marine *Synechococcus* strains (i.e., CC9311, WH8015, WH8016, WH8020, and WH9908), all of which belong to subclade I within marine cluster A. *Synechococcus* WH9908 was isolated in 1999 from Woods Hole, MA, where the water temperature was below 10°C (18), and *Synechococcus* CC9311 was isolated in 1993 from the California Current at a depth of 95 m (6, 16). The genome sequence of coastal strain CC9311 shows a greater tolerance to environmental changes than that of its oceanic counterpart (15, 16). A significant group of picocyanobacteria (members of subclade CB6) living in the Chesapeake Bay in the winter are closely related to CC9311, suggesting that they may have a cold adaptation gene system similar to that of CC9311, which allows them to persist throughout the winter. Further investigations on the cold responses of marine *Synechococcus* CC9311 could shed light on how picocyanobacteria survive in the cold winter. A recent study shows that many *Synechococcus* clones recovered from water samples collected in high latitudes cluster in subclade I, suggesting a possible adaptation of marine *Synechococcus* to low seawater temperatures (31). Members of subclade CB7 overlapped with many environmental clones recovered from brackish water in the Baltic Sea. Currently there are no cultured cyanobacteria from this subclade, but they are likely to represent a group of freshwater-derived cyanobacteria that are able to survive in a cold environment. Garneau et al. (9) detected *Synechococcus* in Arctic waters, but its occurrence seems to be the result of freshwater input (27). Our study suggests that specific groups of picocyanobacteria originating from both marine and freshwater sources can coexist and survive the cold winter season in a temperate estuary.

Conclusion. Although the cell density of picocyanobacteria was low in the winter, the Chesapeake Bay contained two main subclades (CB6 and CB7) of picocyanobacteria in the winter that were not detected in the summer. Subclade CB6 appeared

FIG. 3. CCA of *Synechococcus* communities and environmental factors in the Chesapeake Bay. The plot illustrates the relationship between picocyanobacterial populations and five environmental factors. Among these environmental parameters, only temperature was significantly correlated with picocyanobacterial composition (P , 0.012). The eigenvalues of the x and y axes were 1.000 and 0.961, respectively. The two axes accounted for 49.8% of the observed variation in bacterial composition. P_i , NH_4 , NO_3+NO_2 , and $N:P$ represent the concentrations of phosphate, ammonium, and nitrate plus nitrite and the ratio of N to P, respectively.

to be marine in origin, while subclade CB7 could have been derived from freshwater. Our study showed that temperature was a dominant environmental factor controlling picocyanobacterial populations in the Chesapeake Bay and that specific groups of picocyanobacteria originating from both marine and freshwater sources can coexist and survive the cold winter season in this temperate estuary.

ACKNOWLEDGMENTS

We acknowledge funding support from the National Science Foundation's Microbial Observatories Program (MCB-0132070, MCB-0238515, and MCB-0537041, to F.C.), funds from the Xiamen University 111 program to F.C., and grants 2007CB815904 (Ministry of Science and Technology) and 200805068 (State Ocean Administration) to N.J.

We thank John Hodgkiss for assistance with English.

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