

1 **Title**

2 The first assessment of cyanobacterial and diazotrophic diversities in the Japan Sea

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Abstract

The diversity of cyanobacteria and dizonaotrophs in the Japan Sea was investigated by analyzing sequences of cyanobacterial 16S rRNA genes and nitrogen fixation genes (*nifH*) from seawater sampled at a depth ranging from surface to 100 m at two stations. Of the 107 cyanobacterial 16S rRNA gene sequences obtained, 97 sequences and 3 sequences were assigned to *Synechococcus* sub-cluster 5.1 and *Prochlorococcus* HL (II), respectively. Unlike other oceanic regions, compositional ratio of the sequences assignable to *Synechococcus* sub-cluster 5.3 was relatively high (8%). No sequences of diazotrophic cyanobacteria were found in the cyanobacterial 16S rRNA genes. In *nifH* clone library (36 sequences), 10 sequences were identified as a UCYN-A group of diazotrophic cyanobacteria. Residual 26 sequences (72%) were assigned to proteobacteria. These results suggest heterotrophic bacteria including UCYN-A dominated in diazotrophic community in the Japan Sea. Our study revealed the dominance of *Synechococcus* in cyanobacterial community and (photo)heterotrophic diazotrophs in diazotrophic community in the Japan Sea, suggesting its unique characteristics.

Key words: cyanobacteria, diazotroph, Japan Sea, *Synechococcus* sub-cluster 5.1, *Synechococcus* sub-cluster 5.3, UCYN-A, *nifH*

Introduction

Marine cyanobacteria, especially two genera, *Prochlorococcus* and *Synechococcus*, significantly contribute to 32-80% of the primary productivity in various oceanic regions [1–3]. Some other cyanobacteria retain ability for N₂ fixation which is the process of reducing N₂ gas to biologically available ammonium (referred to as diazotrophic cyanobacteria). The biological N₂ fixation is carried out by several species of bacteria and archaea, but mainly by diazotrophic cyanobacteria in the ocean [4]. Therefore, marine cyanobacteria play an important role in carbon cycle as well as in nitrogen cycle.

Among marine diazotrophic cyanobacteria, filamentous nonheterocystous *Trichodesmium* spp. are the most abundant diazotrophs in the ocean. *Trichodesmium* spp. are observed throughout the tropical and subtropical Atlantic, Pacific, Indian oceans, and East and South China seas [5,6]. In North Atlantic Ocean, their contribution to nitrogen inputs can be as great or greater than nitrate advection from deep sea water [7]. In addition, heterocystous cyanobacterial diatom symbionts such as *Richelia* spp. and *Calothrix* spp. have also been considered to make a significant contribution to N₂ fixation in North Atlantic Ocean and North Pacific Ocean [8,9]. They are detected in tropical and subtropical regions in North Pacific Ocean and North Atlantic Ocean [10,11]. Recently, some unicellular cyanobacteria have been recognized as important diazotrophs in the ocean [12–14]. The unicellular diazotrophic cyanobacteria (UCYN) are phylogenetically sub-divided into three groups, UCYN-A, B and C based on amino acid sequences of NifH (dinitrogenase reductase), a subunit of the nitrogenase complex enzyme. UCYN-A is an uncultivated cyanobacterium lacking oxygenic photosystem II, ribulose biphosphate carboxylase (Rubisco), and tricarboxylic acid cycle, therefore UCYN-A is considered to be photoheterotroph [15].

UCYN-A is shown to be more abundant than other two groups with broad latitudinal range in the North and South Pacific Oceans[10,14]. Unlike UCYN-A, UCYN-B including *Crocospaera watsonii* WH8501 shows a patchy distribution in the ocean, and is detected in South Pacific, Eastern Atlantic, and North Pacific [10,14,16]. Compared to the other two unicellular diazotrophic cyanobacterial groups, UCYN-C including *Cyanothece* TW3 has less abundance in the open ocean [17,18].

The Japan Sea is a semi-enclosed marginal sea in the northwest Pacific surrounded by the Japanese islands and Asian continent. The Japan Sea is often referred to as “the miniature ocean” because the situation, which the course of the western branch of the Tsushima Current and the cold water generated in north and the warm water in east, is similar to those of the global ocean [19]. Its water exchange is limited in the upper layer because the Japan Sea is connected to adjacent seas with the shallow straits [19]. Although many studies have examined the diversity of cyanobacteria in various oceanic regions, less is known about the diversity of cyanobacteria, especially diazotrophic cyanobacteria in the Japan Sea.

In this study, we examined the diversity of cyanobacteria and diazotrophic cyanobacteria vertically in the Japan Sea using polymerase chain reaction (PCR) approach targeting cyanobacterial 16S rRNA genes and *nifH* genes.

Materials and Methods

Seawater collection

Sampling was conducted during Augaust-2011 on board the R/V Tajima at Station 2 (35°50.2N,

134°19.8E, 275 m depth) and Station 7 (37°20.2N, 134°19.8E, 2591 m depth) in the Japan Sea. Seawater was collected in 2l Niskin bottles mounted on a CTD (SBE-9 plus, Sea Bird Electronics) rosette from 0, 10, 30, 75, 100m, and deep chlorophyll maximum (DCM, near 50m). We measured profiles of temperature, salinity, and concentrations of chlorophyll a using the CTD.

DNA extraction

Each 300 ml sample was filtered through 0.2 µm pore-size polycarbonate filters (ADVANTEC, Tokyo, Japan), and then filters were wrapped in aluminum foil and were stored at 4 °C until stored at -80 °C in laboratory. Genomic DNA was extracted from the 0.2 µm filters using ISOIL kit (Nippon Gene, Tokyo, Japan).

PCR amplification

The cyanobacterial 16S rRNA gene sequences were amplified by PCR using primers described by Nübel et al. [20] (Table 1). The genomic DNA sample (1 µl) recovered from each station was added to each reaction mixture. The reaction mixtures were made with 5 µl of 10x PCR Buffer for Blend Taq (TOYOBO, Osaka, Japan), 5 µl of dNTPs (2 mM each), 0.4 µl of forward primer, 0.2 µl of each reverse primer, 0.5 µl of Blend Taq (2.5 U µl⁻¹; TOYOBO), and 37.7 µl of sterile Milli-Q water. After the initial denaturation step (2 min at 94°C), the samples were amplified for 30 cycles (30 s at 94°C, 30 s at 60°C, and 1min at 72°C). To amplify *nifH* genes, we performed nested PCR using the degenerate oligonucleotide primer pair described by Zehr and Turner [21] (Table 1). The reaction mixtures were made with 5 µl of 10x PCR Buffer for Blend Taq (TOYOBO), 5 µl of dNTPs (2 mM each), 2 µl of nifH3, 2 µl of nifH4, 0.5 µl of Blend Taq (2.5 U µl⁻¹; TOYOBO), and 34.5 µl of sterile Milli-Q water. The first

round of PCR consisted of an initial 5 min denaturation at 95°C, then 30 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and final elongation of 7 min at 72°C. Of this reaction product, 1 µl was transferred into a second 50 µl reaction mixture containing the same reagent mixture, and primers were replaced with nifH1 and nifH2. The second round PCR consisted of an initial 5 min denaturation at 95°C, then 30 cycles of 1 min at 94°C, 1 min at 57°C and 1min at 72°C, and final elongation of 7 min at 72°C.

Cloning, Sequencing, and Phylogenetic Analysis

The amplified fragments were purified and cloned into pTac-1 vector (TOYOBO). We sequenced a total of 107 and 36 clones containing cyanobacterial 16S rRNA gene and *nifH* inserts, respectively. Basic Local Alignment Search Tool (BLAST) comparisons were used to find the sequence closely related to those amplified from our samples with the National Center for Biotechnology Information (NCBI) database [22]. The NifH phylogenetic tree based on the deduced amino acid sequences was constructed by the neighbour-joining method with Poisson correction using MEGA version 5 [23].

Nucleotide sequence accession numbers

The 16S rRNA gene sequences and *nifH* sequences reported in this paper have been deposited in the DDBJ under accession numbers AB727363-AB727505.

Results

The vertical thermal stratification was observed in surface water (0-30 m) of both Stn. 2 and Stn. 7 (Fig. 1). The water temperature in surface was relatively constant (25~26 °C) in Stn. 2 and Stn. 7, and water

temperature below 30 m depth declined gradually to 8.2 and 4.5 °C at 200 m depth, respectively (Fig. 1). Salinity of surface water in Stn. 2 was around 32.7 psu, which was a bit lower than that in Stn. 7 (33.3 psu) (Fig. 1). This lower salinity of Stn. 2 was considered to be derived from freshwater input from the terrestrial area. In each station, the concentrations of chlorophyll *a*, the maximum of which was observed around 50 m depth, were extremely low (< 0.2 µM) compared with other oceanic regions [24].

We obtained a total of 107 clones of cyanobacterial 16S rRNA gene sequences from 12 samples in the Japan Sea (Table 2). Of these, 96 sequences (90%) and 3 sequences (3%) were assignable (~99 % nucleotide similarity) to *Synechococcus* sub-cluster 5.1, and *Prochlorococcus* HL-(high-light) (II), respectively. In each station and depth, *Synechococcus* sub-cluster 5.1 dominated the cyanobacterial community. Of the 96 sequences assignable to *Synechococcus* sub-cluster 5.1, 6 sequences showed a 100% identity with *Synechococcus* KORDI-19 which was isolated from the Japan Sea [25]. Residual 8 sequences (8%) were assignable to *Synechococcus* sub-cluster 5.3. This type of sequences was recovered from samples ranging from 10-75 m depth in each station. In general, *Prochlorococcus* has two distinct ecotypes, the high-light(HL)- and low-light(LL)-adapted ecotypes[26]. The sequences similar to a subgroup of the HL ecotype, *Prochlorococcus* HL (II) were obtained only from surface water (0 m), which is comparable with the previous reports that the HL-adapted ecotype generally distributes the upper regions of the euphotic zone [26–28]. No sequences assignable to the LL-*Prochlorococcus* were obtained, suggesting low abundance of LL-*Prochlorococcus* in the subsurface areas (Table 2).

We performed the *nifH*-targeting PCR amplification against the same 12 samples in the Japan Sea (Table 3). We obtained a total of 36 clones of *nifH*. However, no PCR products were detected from DNA

samples of Stn. 2 at DCM, 75 m, 100 m and Stn. 7 at 100 m, suggesting low abundance of diazotrophs in these areas. On the basis of phylogenetic analysis, the *nifH* sequences were divided into five groups (Fig. 2). Sequences assigned to cyanobacteria showed a similarity to the sequences within UCYN-A, and accounted for 28% of the *nifH* clone library. All sequences assignable to UCYN-A were recovered from Stn. 7 at 30m, DCM, and 75m. This result is consistent with the observations that the peak abundances of UCYN-A was observed around 50 m depth in the Pacific Ocean [14]. Other sequences (72%) were grouped within α -, β -, and γ - Proteobacteria. Sequences assigned as α -Proteobacteria (Group 4) showed 100% amino acid identity with sequences recovered from Baltic Sea and Chesapeake Bay (Fig. 2). In β -Proteobacteria, the sequences (Group 3) were recovered from Stn. 2 at 10m and Stn. 7 at 10m with 100% amino acid identity to *Burkholderia vietnamensis* G4 and sequences recovered from North Pacific Ocean. *Burkholderia vietnamensis* were also observed in reservoirs and agricultural settings [29]. Of sequences assigned to γ - Proteobacteria, 12 sequences were highly similar to protein sequences recovered from Atlantic Ocean, South China Sea and Mediterranean Sea.

Discussion

Marine *Synechococcus* is phylogenetically subdivided into three major subclusters, 5.1, 5.2, and 5.3. *Synechococcus* sub-cluster 5.1 dominates *Synechococcus* community in the open ocean [30]. In general, *Synechococcus* are more abundant in eutrophic coastal and mesotrophic open ocean waters with mixing conditions in water column, while *Prochlorococcus* is abundant in oligotrophic waters with stratified

conditions [2,31–33]. Although surface water of the Japan Sea is extremely nutrient-limited environment (eg. under-detection limit for PO₄, NO₃, NO₂) [34], our data showed the dominance of *Synechococcus* in the cyanobacterial community. This discrepancy suggests unknown factors controlling their distribution in the Japan Sea, which will be the subject of future work.

Synechococcus sub-cluster 5.3 accounted for 8% of the clone library with vertical distribution in each station [30, 35, 36] (Table 2). Huang et al. [30] report that their distribution may be restricted to certain oceanic regions because few sequences assignable to *Synechococcus* sub-cluster 5.3 were detected in the GOS database. However, their distribution and characteristics is not well understood.

No sequences belonging to diazotrophic cyanobacteria were detected in the cyanobacterial 16S rRNA gene clone library. It is not surprising because the abundance of diazotrophic cyanobacteria is two to three orders of magnitude lower than that of *Synechococcus* or *Prochlorococcus* in most oceanic regions [10,18,37,38]. In *nifH* clone library, sequences belonging to UCYN-A were obtained from Stn. 7 at 30m, DCM, 75m depths. Due to the elevated phytoplankton biomass around DCM, the carbon source may be more readily available for UCYN-A lacking the carbon fixation enzyme Rubisco. Sequences related to other unicellular cyanobacteria (i.e. UCYN-B and C) were not detected in our *nifH* clone library. Moisander et al. [37] suggest the possibility of limiting the growth of UCYN-A and B by differential grazing pressure due to the smaller cell size of UCYN-A compared to UCYN-B. However, the factors of limiting the distribution of the unicellular diazotrophic cyanobacterial groups have not unraveled yet well.

Sequences of filamentous cyanobacteria were also not detected in our clone libraries (Table 2). Diazotrophic cyanobacteria require a lot of iron as cofactor of nitrogenase and phosphorus for energy

generation during diazotrophic growth [6]. As unicellular cyanobacteria have high surface/volume ratio comparing to filamentous cyanobacteria, they may efficiently grow in oligotrophic environment because phosphate uptake efficiency is dependent upon cell size [39]. Therefore, the presence of UCYN-A and no detection of filamentous cyanobacteria may be due to the extremely nutrient poor condition in the Japan Sea with iron repletion [34].

About two-thirds of *nifH* clones were derived from proteobacteria, and especially sequences most related to γ -Proteobacteria were abundant. Although the sequences belonging to γ -Proteobacteria are detected in various oceans such as North Pacific Ocean and North Atlantic Ocean with accounting for a substantial fraction of the DNA/RNA *nifH* clone libraries, it is still unknown what extent these heterotrophic bacteria contribute to the net nitrogen input in the ocean [40,41]. However, despite the low N_2 fixation rates, heterotrophic bacteria dominated the diazotrophic community in the South Pacific with significant nitrogen input into the ocean [42]. Therefore, given the absence of other diazotrophic cyanobacteria, heterotrophic bacteria may account for substantial fraction of nitrogen input through N_2 fixation in the Japan Sea.

To our knowledge, this is the first study describing molecular cyanobacterial 16S rRNA genes and *nifH* diversity in the Japan Sea. Our data showed wide distribution of *Synechococcus* sub-cluster 5.3 in the Japan Sea vertically. Considering patchy distribution of *Synechococcus* sub-cluster 5.3 in other oceanic regions [30], the Japan Sea may be one of the suitable environments for them to propagate. The dominance of *Synechococcus* with oligotrophic well-stratified condition environment was contradict to the general distribution of cyanobacteria in the ocean, therefore these results may reflect the unique

characteristics in the Japan Sea. We also showed that heterotrophic bacteria dominate the diazotrophic community. Further studies are required for determining what factors affect the vertical and horizontal distributions of different groups of these microorganisms in the Japan Sea.

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Figure Legends

Figure 1

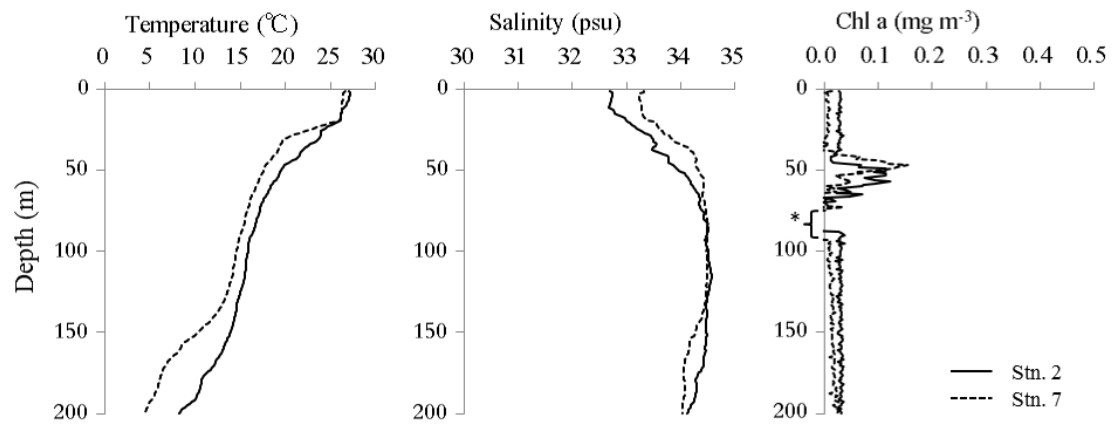
Vertical distributions (0-200 m) of temperature, salinity, chlorophyll *a* concentrations (mg m⁻³) at Stn. 2 (solid line) and Stn. 7 (dotted line) in Japan Sea on August 2011. The asterisk shows that fluorescent intensity was below zero.

Figure 2

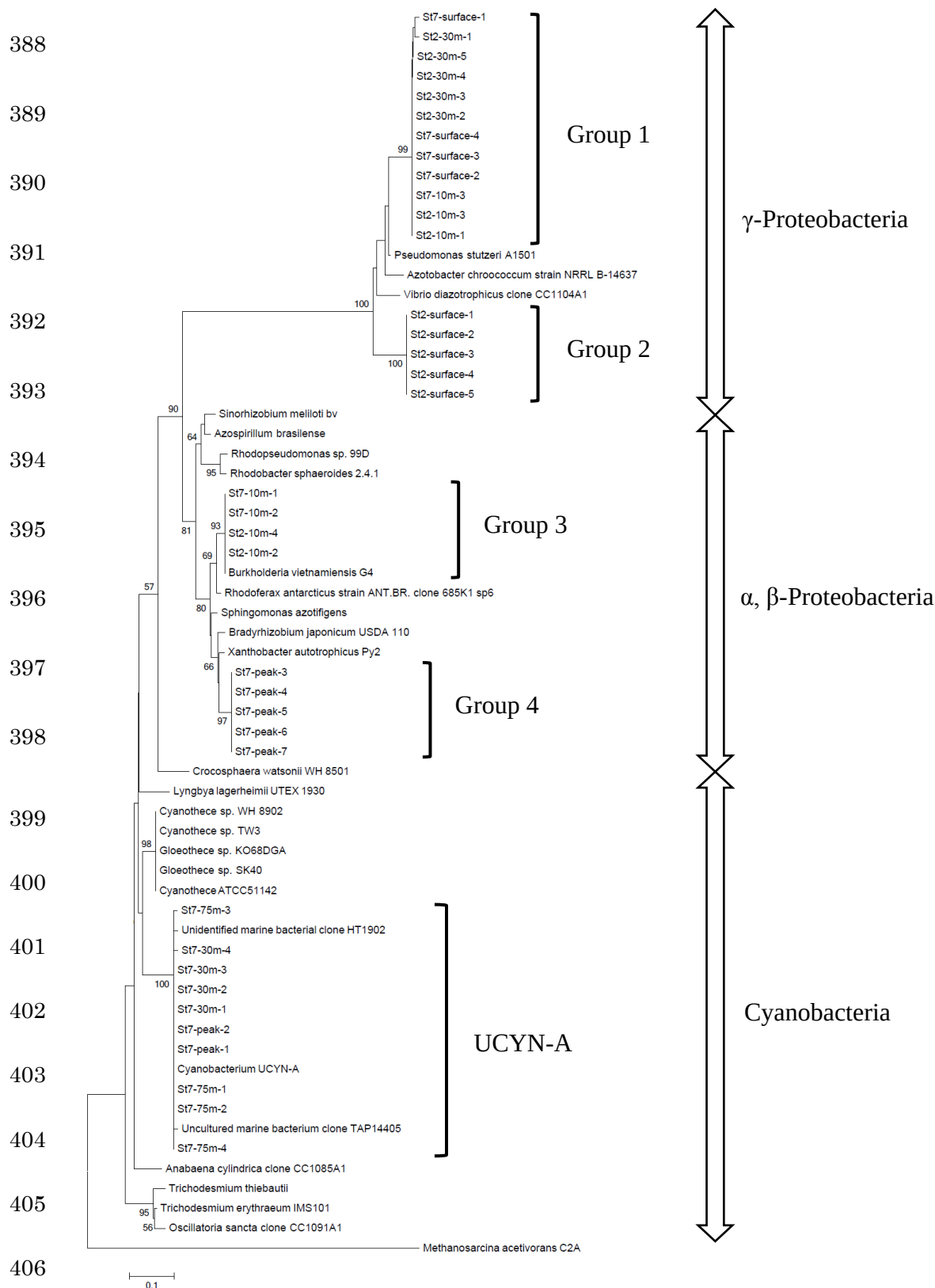
Phylogenetic trees for *nifH*-deduced amino acid sequences from each sample constructed using the neighbor-joining method. Bootstrap values greater than 50% for 1000 replicates are indicated at respective nodes. Database entries for sequences are as follows: *Azotobacter chroococcum* strain NRRL B-14637 (AAR11505); *Pseudomonas stutzeri* A1501 (YP_001171863); *Vibrio diazotrophicus* clone CC1104A1 (AAP48983); *Sinorhizobium meliloti* bv (ABC67302); *Azospirillum brasilense* (P17303); *Rhodopseudomonas* sp. 99D (BAC07290); *Rhodobacter sphaeroides* 2.4.1 (YP_353614); *Burkholderia vietnamiensis* G4 (YP_001115195); *Rhodferax antarcticus* strain ANT.BR. clone 685K1_sp6 (ABI14697); *Sphingomonas azotifigens* (BAE71134); *Bradyrhizobium japonicum* USDA 110 (BAC47034); *Xanthobacter autotrophicus* Py2 (YP_001415004); *Crocospaera watsonii* WH 8501 (AAP48976); *Anabaena cylindrica* clone CC1085A1 (AAP48968); *Lyngbya lagerheimii* UTEX 1930 (AAA19029); *Cyanothece* sp. ATCC51142 (AAB61408); *Gloeotheca* sp. SK40 (BAF51659); *Gloeotheca* sp. KO68DGA (BAF51663); *Cyanothece* sp. WH 8902 (AAV28035); *Cyanothece*

sp. TW3 (BAJ21365); Unidentified marine bacterial clone HT1902 (AAK83129); Uncultured
marine bacterium clone TAP14405 (AAY60051); Cyanobacterium UCYN-A (ACJ53724);
Oscillatoria sancta clone CC1091A1 (AAP48970); *Trichodesmium thiebautii* (AAA77023);
Trichodesmium erythraeum IMS101 (AAA77022); *Methanosarcina acetivorans* C2A (NP_616144)

Hashimoto et al., Figure 1



Hashimoto et al., Figure 2



407 Table 1. Cyanobacterial 16S rRNA and *nifH* primers used in this study.

Primer	Target site	Sequence(5'-3')	Reference
CYA359F	16S rRNA of cyanobacteria	GGG GAA TYT TCC GCA ATG GG	Nübel et al. 1997
CYA781R(a)	16S rRNA of cyanobacteria	GAC TAC TGG GGT ATC TAA TCC CAT T	Nübel et al. 1997
CYA781(b)	16S rRNA of cyanobacteria	GAC TAC AGG GGT ATC TAA TCC CTT T	Nübel et al. 1997
nifH1	<i>nifH</i> of prokaryotes	TGY GAY CCN AAR GCN GA	Zehr and McReynolds 1989
nifH2	<i>nifH</i> of prokaryotes	AND GCC ATC ATY TCN CC	Zehr and McReynolds 1989
nifH3	<i>nifH</i> of prokaryotes	ATR TTR TTN GCN GCR TA	Zani et al. 2000
nifH4	<i>nifH</i> of prokaryotes	TTY TAY GGN AAR GGN GG	Zani et al. 2000

Table 2. Summary of cyanobacterial 16S rRNA gene clones obtained from each microbial group.

Station	Depth	Number of Clones			Total
		<i>Synechococcus</i> sub-cluster 5.1	<i>Synechococcus</i> sub-cluster 5.3	<i>Prochlorococcus</i> HL(II)	
2	0m	7		2	9
	10m	9	1		10
	30m	9	1		10
	DCM	8	2		10
	75m	8			8
	100m	9	1		10
7	0m	9		1	10
	10m	10			10
	30m	9	1		10
	DCM	9	1		10
	75m	9	1		10
	100m				-

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435 Table 3. Summary of *nifH* clones obtained from each microbial group.

Station	Depth	Number of Clones			Total
		Cyanobacterium UCYN-A	α -, β - Proteobacteria	γ - Proteobacteria	
2	0m			5	5
	10m		2	2	4
	30m			5	5
	DCM				-
	75m				-
	100m				-
7	0m			4	4
	10m		2	1	3
	30m	4			4
	DCM	2	5		7
	75m	4			4
	100m				-

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