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Vertical distribution and phylogenetic composition of bacteria in the Eastern Tropical North Pacific Ocean

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Summary

The vertical community structure of bacteria along a depth profile in the Eastern Tropical North Pacific Ocean (13°N, 104°W) was studied by flow cytometry measurement and 16S rRNA gene clone libraries analysis. Picoeukaryotes and *Synechococcus* peaked at 30 m and decreased sharply below 50 m, while *Prochlorococcus* peaked at both 30 and 100 m layers and disappeared below 200 m. Heterotrophic bacteria peaked above shallow thermocline and decreased along the depth profile. Sequences of total 322 clones from four clone libraries (10, 100, 1000, and 3000 m) clustered into nine major lineages. γ-Proteobacteria dominated all the depths and occupied almost the whole bacterial community at the 3000 m. α-Proteobacteria was abundant throughout the water column except near the sea bottom, and δ-Proteobacteria peaked at the 1000 m depth. Cyanobacteria were primarily limited to the photic zone, and the genetic diversity of *Prochlorococcus* showed a good correlation with niche adaptation. The appearance of the *Cytophaga–Flexibacter–Bacteroides* (CFB) group did not show a clear relationship with depth. *Actinobacteria* were found both in the photic zone and in deep water. *Planctomyetes*, *Acidobacteria*, and *Verrucomicrobia* were present as minor groups and more dominant in the deeper layers of water.

Introduction

Bacteria are thought to be important components of aquatic ecosystems and play a critical role in the global carbon cycle (Cole et al. 1988).

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Although they are all unicellular and similar in cell size, bacteria are composed of different taxonomic groups with potentially different phenotypic properties, physiological activities, and ecological functions. The connection between overall bacterial community structure and environmental variables is critical for understanding the function of various bacteria within marine carbon and nutrient cycles. In aquatic systems, one of the most relevant gradients affecting the community structure is depth in water columns. Molecular methods targeting 16S rRNA genes have revealed some details of the bacterial phylogenetic diversity from different depths in the northeast Pacific, the subtropical and temperate Atlantic Oceans (Delong et al. 2006; Gallagher et al. 2004), and in the Antarctic Polar Front (López-García et al. 2001). These results indicated that the deep sea contains numerous novel and widespread major prokaryotic lineages. Using rRNA hybridization and gene cloning and sequencing, García-Martínez et al. (2002) found that *Alteromonas macleodii*-like microorganisms are present in high proportions in deep waters, suggesting that some specific bacteria might play an important ecological role in deep waters. The progress, however, is far from complete. A recently reported study has shown that bacterial communities of deep water masses of the North Atlantic are far more complex than previously reported for any microbial environment (Sogin et al. 2006).

Compared with the oligotrophic North Atlantic and the subtropical gyre in the western Pacific, the Eastern Tropical North Pacific (ETNP) exhibits a higher surface nutrient level, chlorophyll concentrations, and higher biological productivity (Schlitzer 2004). The eutrophic environment might support a bacteria community different from the oligotrophic oceans. In the present study, we examined four seawater samples obtained from different depths in the ETNP. 16S rRNA gene clone libraries were constructed and a total of 332 sequences were obtained and subject to a detailed analysis, with the aim to better characterize the distribution pattern of bacterial community along the depth profile.

Materials and methods

Sample collection

Water samples for flow cytometry analysis were collected at 1, 10, 30, 50, 75, 100, 125, 150, 175, 200, 500, 1000, 1500, 3000, 3100, and 3150 m depths using Niskin bottles (Oceanic) in the ETNP (13°N, 104°W) in November 2003. Samples were fixed with glutaraldehyde (final concentration: 1%), quick-frozen in liquid nitrogen, and stored in a freezer at −20 °C for later analysis. Five liter seawater samples collected from 10, 100, 1000, and 3000 m (about 150 m above the sea bottom) depths for clone libraries construction were filtered onto 47-mm diameter 0.2 μm-pore-size polysulfone filters (PALL, Ann Arbor, MI, USA) at a pressure <0.03 MP. Filtered samples were immediately frozen and stored at −20 °C until DNA extraction.

Data for temperature, salinity, and chlorophyll were from the Chinese JGOFS cruise project reports of the same cruise.

Flow cytometry measurement

Glutaraldehyde-fixed samples were run on a FACS Calibur flow cytometer (Becton-Dickinson), equipped with an external quantitative sample injector (Harvard Apparatus PHD 2000). Procedures were as described by Marine et al. (1997). The three autotrophs (*Prochlorococcus*, *Synechococcus*, and picoeukaryotes) and heterotrophic bacteria were distinguished and enumerated according to Jiao et al. (2002).

DNA extraction, PCR amplification, and clone library construction

Total DNA was extracted and purified according to the previously described method (Gallagher et al. 2004) and was used for PCR amplification of the 16S rRNA genes. The universal bacterial primers, 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GGTACCTTGTTACGAC TT-3′), were used for PCR amplification, and the amplification procedures were the same as Bano and Hollibaugh (2002). The amplified products were gel-purified and ligated into the pMD18-T vector (TaKaRa, Dalian, China) and then transformed into competent cells of *Escherichia coli* DH5α. The ampicillin-resistant clones were randomly picked and screened for inserts by performing colony PCR with M13 primers (Invitrogen, Shanghai, China) for the vector.

DNA sequencing, statistical analysis of the clone libraries, and phylogenetic tree construction

A total of 337 clones with correct inserts (approximately 1500 bp) were directly sequenced. The sequencing was carried out on an ABI model 377 automated DNA sequence analyzer (Applied

Biosystems, Perkin-Elmer) using the primer 27F. All the nucleotide sequences were checked for putative chimeras by the CHIMERA_CHECK (Maidak et al. 2001), and reliable sequences were compared to known 16S rDNA sequences in the database by using the BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were then assigned to major groups by using BLAST similarities and RDP Classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp). Sequences were also grouped as operational taxonomic units (OTUs) by 97% or greater sequence similarity in the DOTUR program (Schloss and Handelsman 2005). Diversity indices (Coverage, Dominance, Evenness, Shannon, and Simpson) were calculated using the statistical program PAlaeontological STatistics (PAST, ver. 1.34, http://folk.uio.no/ohammer/past). Representative sequences from each OTU were aligned using the software ClustalX (Thompson et al. 1997), and phylogenetic trees were constructed by the software MEGA3 using the neighbor-joining algorithm (Kumar et al. 2004).

Nucleotide sequence accession numbers

Clone sequences have been deposited in GenBank under the accession numbers AY664161 to AY664249, AY726783 to AY726875, AY726876 to AY726979, and AY726980 to AY727030.

Results

Depth profile of hydrographic and biological variables

The depth profiles of picoplankton cell abundance, temperature, salinity, and chlorophyll *a* of the water column are shown in Figure 1. Salinity was stable throughout the water column, while a thermocline was between 30 and 75 m, separating the surface mixed layer at a temperature of 27 °C from the water below 75 m at a temperature of 16 °C. In addition, a much deeper thermocline was present at depths between 200 and 1000 m, marking the change from cold (>11 °C) to very cold water (<5 °C, Figure 1). The chlorophyll *a* concentration was high at depths above 100 m (ranging from 0.14 to 0.28 μg/L), peaked at 30 m (0.28 μg/L) and 100 m (0.26 μg/L) depths, and then declined dramatically with depth to nearly under detection limits (⩽0.003 μg/L) at depths below 175 m. Correspondingly, the picoplankton cell abundance showed a good depth-related as well as temperature-related distribution pattern. The autotrophic picoplankton (picoeukaryotes, *Prochlorococcus*, and *Synechococcus*) peaked at 30 m, decreased sharply below the first thermocline (except for the *Prochlorococcus*, it had another peak at about 100 m) and disappeared below 200 m. The heterotrophic bacteria also peaked above the first thermocline and decreased sharply below 50 m and then gradually with depth. They had another dramatic decline below the second thermocline but could be detected throughout the water column.

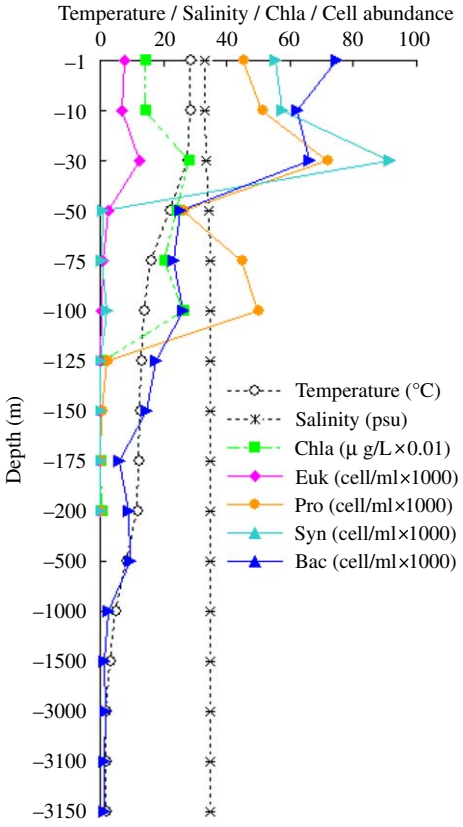


Figure 1. Depth profiles of temperature, salinity, chlorophyll *a*, and picoplankton cell abundance in the Eastern Tropical North Pacific Ocean (13°N, 104°W). Euk: picoeukaryotes; Pro: *Prochlorococcus*; Syn: *Synechococcus*; Bact: heterotrophic bacteria.

Statistical analysis of 16S rDNA clone libraries

A total of 337 clones with correct inserts were sequenced and the potential chimeras were removed. There were 322 reliable sequences (85 from 10 m, 88 from 100 m, 99 from 1000 m, and 50 from 3000 m) used for further analysis. These cloned sequences were designated as JL-ETNP-H (or I, R, S, Y, Z, E, and F) *n*, in which H and I represent clones that are derived from 10 m depth, R and S from 100 m, Y and Z from 1000 m, E and F from 3000 m

depth, and *n* represents the number of the clone. Sequences were grouped into 44, 42, 59, and 23 OTUs in each clone library, and the diversity indices, the *Shannon* and *Simpson* are both highest in the 1000 m depth (3.74 and 0.96, respectively) and lowest in the 3000 m depth (2.51 and 0.83, respectively), indicating that the overall diversity was highest in 1000 m and lowest in 3000 m. The highest *Dominance* occurred in the 3000 m (0.17), corresponding to its lowest *Evenness* (0.53) among the depth layers, indicating that the bacterial community near the sea bottom was comprised by a few phylotypes. The second highest *Dominance* was in the deep chlorophyll maxima of 100 m depth (0.08), corresponding to its lower *Evenness* (0.58) than that of the other two depths (about 0.7 in the 10 and 1000 m), suggesting that the deep chlorophyll maxima layer might grow some specific phylotypes. However, the values of coverage of the clone libraries were not high (ranging from 53.54% to 68.18%), indicating that the diversity might have been underestimated.

Depth profile of the composition of 16S rDNA clone libraries at division and subdivision levels

The bacterial community structure was analyzed by the 16S rDNA clone library method in detail, and the species-composite clone libraries are shown in Figure 2. All of our sequences fell into nine major lineages of the bacterial domains: α (18%), γ (58%), and δ-Proteobacteria (10%); cyanobacteria (*Prochlorococcus* and *Synechococcus*, 6%); *Cytophaga–Flexibacter–Bacteroides* group (CFB, 2%); and *Actinobacteria*; *Planctomycetes*, *Acidobacteria*, and *Verrucomicrobia* as well as some clones originating from eukaryotic chloroplast or plastids. The percentage of 16S rDNA sequences of each group in the total picoplankton rDNA pool indicated that the γ-Proteobacteria dominated all the depths and occupied almost the whole picoplankton community (84%) at 3000 m, corresponding to the highest *Dominance* of the depth layer. α-Proteobacteria was abundant (17–26%) throughout the water column except for the sea bottom (4%). δ-Proteobacteria peaked (27%) at the 1000 m depth layer, but occupied a minor component at shallower water (1–2%) and bottom of the sea (4%). The abundance of autotrophic picoplanktons showed similar distribution patterns as seen through the FCM enumeration. The 16S rDNA sequences of eukaryotic chloroplast or plastid were only detected in shallow water. *Prochlorococcus* were more abundant at the 100 m than that of the 10 m, while *Synechococcus* showed a contrary distribution pattern. *Synechococcus* were more abundant in the surface mixed layer, decreased gradually with depth, and showed a deeper distribution than that of *Prochlorococcus*.

A few high G+C Gram-positive bacteria (*Actinobacteria*) were only detected at the middle two depths, 100 and 1000 m, and were mainly distributed at 100 m. The CFB group was found throughout the water column except at the 100 m depth, and its abundance did not show a clear relationship with depth. *Verrucomicrobia*, *Acidobacteria*, and *Planctomycetes* were also detected as minor groups and tended to be more abundant at deeper water layers.

Phylogenetic analysis of each group

α-Proteobacteria

A total of 57 sequences (18%) grouped with the α subdivision of the proteobacteria, dominated by SAR11 sequences (31 of 57). The SAR11 cluster, assigned to 22 OTUs, could be further subdivided

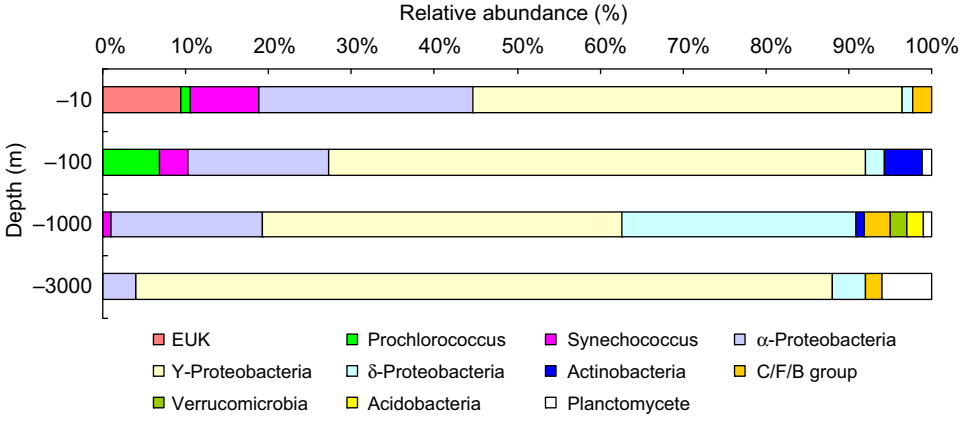
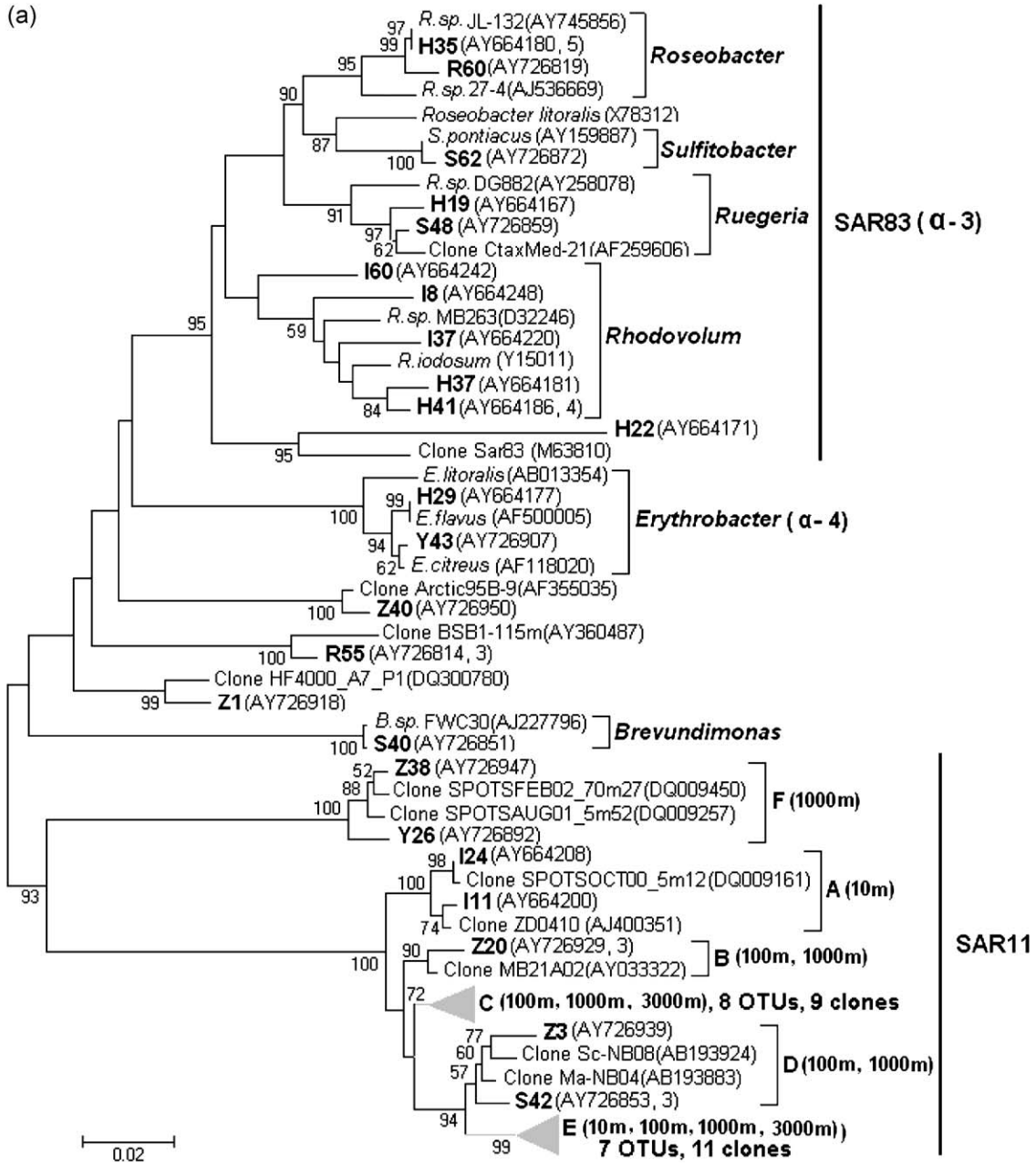
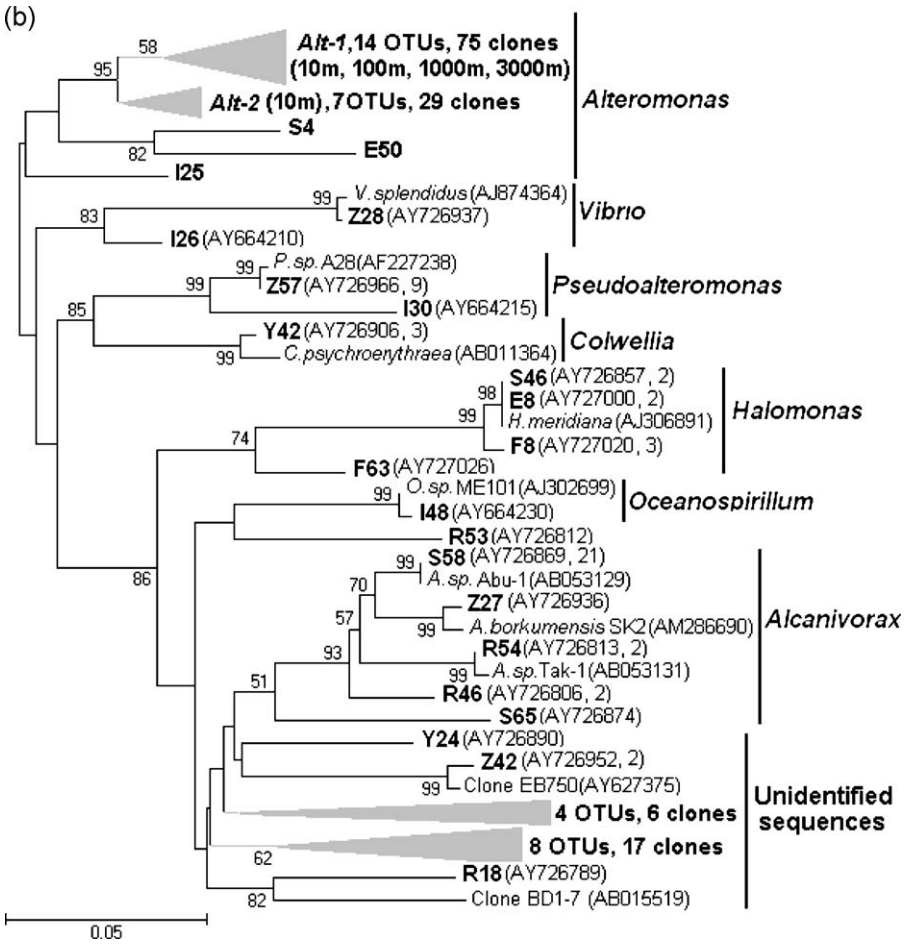


Figure 2. Percentage of abundance of the bacterial phylotypes as determined by 16S rDNA clone library statistics and phylogenetic analysis. EUK: eukaryotic-like clones; C/F/B group: *Cytophaga–Flexibacter–Bacteroides* group.

into six subclusters (A–F, Figure 3a), and four of which (A–D) showed different depth-related distributions. All the clones in subcluster A were from the 10 m depth, while all the clones in subclusters B–D were found at $\geqslant$100 m depth layers and were most closely related with clones obtained from deep waters of various environments. Sequences in subcluster E had no clear relationship to the



Figures 3a–c. Neighbor-joining trees generated from alignments of 16S rDNA sequences from the ETNP samples and representative references retrieved from Genbank. Some groups in the trees were compressed using Mega 3.0 program with vertical height and horizontal width of the triangles representing OTUs abundance and degree of divergence, respectively, within a given group. Accessions numbers are shown in parentheses. Clones from this study are indicated in boldface and designated as H (or I, R, S, Y, Z, E, and F) *n*, in which H and I represent clones that derived from 10 m depth, R and S from 100 m, Y and Z from 1000 m, E and F from 3000 m depth, and *n* represents the number of different clones. Numbers in brackets that follow the accession numbers indicate the occurrence frequency of the OTUs in corresponding libraries. Bootstrap values above 50 (100 iterations) are shown at each node. Scale bars represent the nucleotide substitution percentage. (a) α-Proteobacteria. (b) γ-Proteobacteria. (c) Others. HL and LL refer to high-light-adapted and low-light-adapted genotypes of *Prochlorococcus*, respectively.



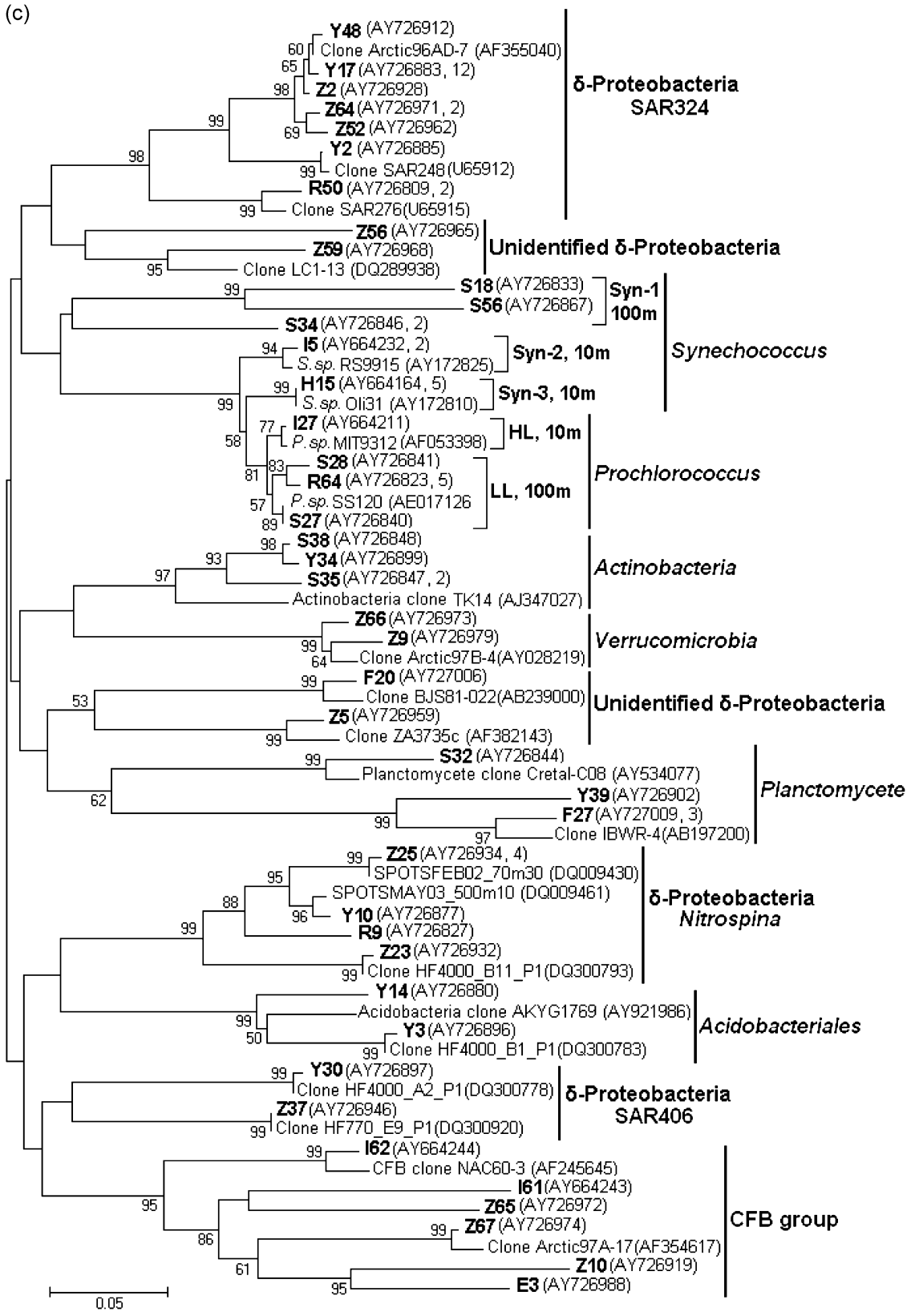
Figures 3a–c. (*Continued*)

sampling depth. They were found throughout the water column and were most similar to clones retrieved from different depths (Figure 3a). Two 1000 m clones (Y26 and Z38) affiliated with SAR11 cluster (93% bootstrap support) formed a separate subcluster F and clustered with clones from different depths of Southern California offshore.

The second most abundant cluster within the α-Proteobacteria was the SAR83 (α-3) cluster (Allgaier et al. 2003; Beja et al. 2002), they were all retrieved in the upper layers of the ocean ($\leqslant$100 m depth), and were clustered with the *Rhodovulum*, *Ruegeria*, *Roseobacter*, and *Sulfitobacter* genera and the SAR83 clone (Figure 3a). Two sequences (Y43 and H29) clustered tightly with the typical aerobic anoxygenic phototrophic bacterium *Erythrobacter litoralis*, and composed the α-4 subclass of proteobacteria (Figure 3a). Only one clone (S40) was grouped into *Brevundimonas* genus. Three other clones (Z40, R55, and Z1) contained inserts that were closely related to only environmental sequences (Figure 3a).

γ-Proteobacteria

The highest diversity of phylotypes was found within the γ-Proteobacteria. A total of 186 sequences (58%), classified into 55 OTUs, were clustered into the γ-Proteobacteria. These belonged to seven genera and a few environmental sequences (Figure 3b). Clones affiliated with *Alteromonas* were dominant groups (56% of all sequences identified as γ-Proteobacteria) and were mainly composed of two subclusters (*Alt*-1 and 2). Sequences in *Alt*-1 were obtained from different depth layers, while sequences in subclusters *Alt*-2 could only be found in the 10 m depth. *Alt*-1 was the most frequently occurring phylotype, it comprised 14 OTUs and 75 clones and was closely affiliated with *A. macleodii* strains and some attached bacteria. *Alt*-2 comprised 7 OTUs and 29 clones and was most closely related to some unclassified bacteria, including a biphenyl-degrading bacterium. Three additional *Alteromonas*-affiliated clones had no close relatives in the GenBank and could not group with any known sequences in the database (Figure 3b).



Figures 3a–c. (*Continued*)

Other γ-affiliated clones were grouped into the *Alcanivorax* (27 clones), *Pseudoalteromonas* (10 clones), *Halomonas* (eight clones), *Colwellia* (three clones), *Vibrio* (two clones), and *Oceanospirium* (one clone), respectively (Figure 3b), and most of these clones (94%) were obtained from $\geqslant$100 m depths. These clones were mainly related with some functionally important bacteria, such as the hydrocarbon- or petroleum-degrading bacteria, the alga-lysing bacteria, and some extremely halotolerant or psychrophilic bacteria (Figure 3b).

The remaining γ-affiliated clones (27 clones, 15 OTUs) were only related to unidentified sequences, including the OM182 clade and the SAR86 cluster sequences. All of the clones were obtained at $\geqslant$100 m depths and most of them were closely related with clones derived from deeper-layer waters, suggesting that a lot of novel bacteria not previously recognized exist in deep layers of the ETNP Ocean.

δ-Proteobacteria, Cyanobacteria, and others

A total of 33 clones (11%) classified into 17 OTUs were affiliated with the δ-Proteobacteria, which comprised SAR324, SAR406, and *Nitrospina* subclusters and two unidentified subclusters (Figure 3c). Subclusters SAR324 and *Nitrospina* were predominant (containing 20 and 7 clones, respectively), while the others contained only one to two clones, respectively (Figure 3c).

A total of 19 sequences (6% of all sequences we obtained), assigned to nine OTUs, were affiliated with cyanobacteria, in which eight sequences were *Prochlorococcus*, and others were *Synechococcus*. Clone I27 was obtained from 10 m depth and was most similar to the high-light-adapted ecotype (HL) *Prochlorococcus* strain, while all the other *Prochlorococcus* clones were from 100 m depth and were most closely related to the low-light-adapted ecotype (LL) strains, which showed a good correlation between genetic diversity and ecotypic adaptation (Figure 3c). *Synechococcus*-affiliated clones also showed a depth-related difference of distribution. Clones derived from the 100 m depth composed a separate subcluster (Syn-1) and could not be grouped meaningfully with any known *Synechococcus*, suggesting that these clones represent some novel *Syenchococcus* not previously recognized. Clones derived from the 10 m depth were subdivided into two subclusters (Syn-2 and Syn-3) and were most closely related to strains isolated from the Red Sea and the equatorial Pacific, respectively (Fuller et al. 2003).

Six clones (about 2%) were affiliated with the CFB group. These clones were obtained from different depth layers and were closely related with some functionally important bacteria, such as the organic matter-degrading bacterium *Leeuwenhoekiella blandensis* (Pinhassi et al. 2006), the amoebae-resisting bacterium *Candidatus Amoebinatus massiliae* (Greub et al. 2004), and some uncultured CFB-related clones from different depths of various marine waters. The remaining 13 clones were grouped into the *Planctomycetales* (five clones), the *Actinobacteria* (two clones), the *Verrucomicrobium* (two clones), and the *Acidobacteriales* group (two clones, Figure 3c).

Discussion

Analysis of bacteria diversity and dominant group in the ETNP water column

Statistical analysis of the clone libraries showed that the diversity of 1000 m depth clone library was highest among the libraries. Further analysis revealed that the highest diversity was mainly due to high abundance of the δ-Proteobacteria, which contributed 14 OTUs to the library, while only one to two OTUs to the other three libraries (Figures 2 and 3c). Another reason might lie in the presence of the minorities of *Verrucomicrobia* and *Acidobacteriales*. They were detected only in this library and contributed four OTUs to the diversity (Figures 2 and 3c). The 3000 m depth was charactered by its lowest diversity and highest *Dominance*, and the *Dominance* was far higher (0.17) than the other depth libraries (0.04–0.08). The most abundant phylotype in this depth occurred 19 times and occupied 38% of the clone library, and was closely related to the *A. macleodii*. The organisms within *A. macleodii* are prevalent in different oceanic regions and seem to be more frequent in deep waters (García-Martínez et al. 2002). This kind of organisms is thought to be a strategist that can grow rapidly when organic nutrients are readily available (López-López et al. 2005). These characteristics provide *A. macleodii* advantages when competing with other bacteria in extreme environments, such as the deep sea in our study. However, temperature was thought to be one of the factors that might preclude *A. macleodii* since these organisms were absent in cold latitudes (García-Martínez et al. 2002). Nevertheless in our case, *A. macleodii*-like clones were found to be predominant down to the 3000 m depth layer where the temperature was only 1.8 °C, though the total bacterial abundance was very low there (Figure 1).

The second highest dominance occurred at the 100 m depth, and was due to the abundant clones

related to *Alcanivorx* spp. and also *A. macleodii*. They occupied 22.7% and 12.5%, respectively, in the clone library. Since *Alcanivorax* spp. uses oil hydrocarbons as their exclusive source of carbon and energy and is barely detectable in unpolluted environments (Schneiker et al. 2006), there probably exists hydrocarbon pollution in this layer. The abundant presence of *A. macleodii* confirmed its widespread distribution in marine habitats.

Variation of different phyla along the depth profile

Phylogenetic analysis of 16S rDNA clones from the ETNP revealed significant diversity among the cloned sequences, with similarity to previously reported sequences ranging from 86% to 99%. Most of the sequences showed depth-specific distribution along the vertical profile. For example, sequences in the α-3 (SAR83) phylotype tended to be limited to the upper ocean, while the α-4 (*Erythrobacter*)-affiliated clones were less abundant but could distribute deeper than the α-3 (Figure 3a). Even in the ubiquitous SAR11 cluster, there existed different highly depth-specific distributions (Figure 3a and Field et al. 1997). As for the γ-Proteobacteria, the *Alteromonas* dominated all depths, but the subcluster *Alt*-2 was mainly distributed in the 10 m depth. Although the 16S rDNA sequence is much too stable to allow the identification of depth-related ecotypes, a previous work carried out with housekeeping genes did show that there exists a deep-sea ecotype of *A. macleodii* (López-López et al. 2005). Clones related to the *Alcanivorax* peaked at the 100 m depth, but were absent or just a minor component at other depths. *Halomonas* were relatively more abundant at deeper oceanic waters than the shallow water, and *Colwellia*, *Vibrio*, and *Oceanospirillum* were only found in certain samples, which indicated that the distributions and abundance of many genera varied with depth. Proteobacteria in the δ-subdivision also display uneven distribution in aquatic environments. SAR324 is vertically stratified and peaked at certain depths in the water columns of both the Atlantic and Pacific oceans (Wright et al. 1997), which is generally in agreement with our results (peaking at 1000 m in the ETNP, Figure 2). These suggest that microbial communities in the lower surface layers of this region may be functionally specialized.

The vertical patterns of *Prochlorococcus* and its ecotypes suggest causal relationships with environmental variables. Light and temperature are two important variables shaping *Prochlorococcus* distribution. As has been observed recently in the Atlantic Ocean (Johnson et al. 2006), the HL clade MIT9312 dominating near the surface grew faster at a temperature of 25 °C, but could not grow at temperatures below 15 °C, while the LL clade MIT9313 peaked at 100 m, and was more abundant in waters between the 13 and 23 °C isotherms and was near the limit of detection at temperature above 25 °C. Similarly, our ETNP clone within the MIT9312 clade appeared at 10 m where the temperature was 28 °C, and disappeared at 100 m where the temperature was 14 °C, while the clones within MIT9313 clade were derived from the 100 m depth (13.8 °C) and were not found in the 10 m depth (28 °C) (Figures 1 and 3c).

Other important phyla also show uneven vertical distribution, not only in this case but also in other environments (Nold and Zwart 1998). *Actinobacteria* have been found both in the photic zone and in deep water, and seem to be more frequently recovered in deep water (Delong et al. 2006), but our *Actinobacteria*-like clones seemed to be more abundant in the photic zone (Figure 2). *Verrucomicrobia*-affiliated sequences had been found in shallow waters (10–200 m) of North Pacific Ocean (Delong et al. 2006), while our study extended its distribution down to the 1000 m depth layer (Figure 2). Members of *Acidobacteria* and *Planctomycete* were mainly recovered from deep waters (Figure 2), which was in agreement with previous studies (Delong et al. 2006).

The repeated discovery of sequences belonging to different gene clusters with similar distributions in the water column from different marine habitats suggests that the vertical variable in the water column is one of the most relevant gradients affecting the distribution of microorganisms. The reason for microbial variations is not fully understood, but the stratified microbial communities and the phylogenetic analysis described in this study will be good supplements to the current understanding of biogeographic distribution of bacterioplankton and will provide important foundational data for future studies on microbial functionality.

Acknowledgments

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