

Impact of redox-stratification on the diversity and distribution of bacterial communities in sandy reef sediments in a microcosm*

GAO Zheng (高峥)¹, WANG Xin (王鑫)², Angelos K. HANNIDES², Francis J. SANSONE², WANG Guangyi (汪光义)^{2,3,**}

¹ State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Tai'an 271000, China

² Department of Oceanography, University of Hawaii, 1000 Pope Road, Honolulu, Hawaii 96822, USA

³ Shenzhen Engineering Laboratory for Algal Biofuel Technology Development and Application, School of Environment and Energy, Peking University Shenzhen Graduate School, Shenzhen 518055, China

Received Dec. 25, 2010; accepted in principle Feb. 15, 2011; accepted for publication Apr. 7, 2011

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Abstract Relationships between microbial communities and geochemical environments are important in marine microbial ecology and biogeochemistry. Although biogeochemical redox stratification has been well documented in marine sediments, its impact on microbial communities remains largely unknown. In this study, we applied denaturing gradient gel electrophoresis (DGGE) and clone library construction to investigate the diversity and stratification of bacterial communities in redox-stratified sandy reef sediments in a microcosm. A total of 88 Operational Taxonomic Units (OTU) were identified from 16S rRNA clone libraries constructed from sandy reef sediments in a laboratory microcosm. They were members of nine phyla and three candidate divisions, including *Proteobacteria* (*Alpha*-, *Beta*-, *Gamma*-, *Delta*-, and *Epsilonproteobacteria*), *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Verrucomicrobia*, *Spirochaetes*, and the candidate divisions WS3, SO31 and AO19. The vast majority of these phylotypes are related to clone sequences from other marine sediments, but OTUs of *Epsilonproteobacteria* and WS3 are reported for the first time from permeable marine sediments. Several other OTUs are potential new bacterial phylotypes because of their low similarity with reference sequences. Results from the 16S rRNA, gene clone sequence analyses suggested that bacterial communities exhibit clear stratification across large redox gradients in these sediments, with the highest diversity found in the anoxic layer (15–25 cm) and the least diversity in the suboxic layer (3–5 cm). Analysis of the *nosZ*, and *amoA* gene libraries also indicated the stratification of denitrifiers and nitrifiers, with their highest diversity being in the anoxic and oxic sediment layers, respectively. These results indicated that redox-stratification can affect the distribution of bacterial communities in sandy reef sediments.

Keyword: bacterial diversity; bacterial stratification; biogeochemical gradients; sandy reef sediments

1 INTRODUCTION

Marine microbial communities are fundamental regulators of biogeochemical cycles, and their relationships with geochemical environments are at the center of marine microbial ecology and biogeochemistry (Falkowski et al., 2008; Strom, 2008). Although several molecular finger-printing methods have been applied to the investigation of sandy sediment microbial communities (Llobet-Brossa et al., 1998; Mills et al., 2003; Rusch et al., 2003; Buhning et al., 2005; Hewson et al., 2006;

Hunter et al., 2006; Sørensen et al., 2007; Mills et al., 2008), our understanding of the impact of abiotic factors on microbial communities in sandy sediments remains limited. In particular, although biogeochemical redox stratification has been well

* Supported by a NOAA Grant (No. NA04OAR4600196 (GW)), the microcosm development and operation was supported by the U. S. National Science Foundation (Nos. OCE03-27332 and OCE05-36616 (FJS)), and a project of Shandong Province Higher Education Science and Technology Program (No. J10LC09)

** Corresponding author: gywang@pkusz.edu.cn, guangyi@hawaii.edu
GAO Zheng and WANG Xin contribute equally to this work.

documented in marine sediments (Canfield et al., 1993; Thamdrup et al., 1994), information on the correlation of microbial community composition with redox stratification in sediments, particularly marine sandy sediments, remains largely unknown (Urakawa et al., 2000; Edlund et al., 2008). One of the key questions is whether major functional microbial groups are evenly distributed in different sediment redox layers due to active advective exchange in sandy sediments, or if redox gradients influence microbial distribution.

Sandy reef sediments, which are widespread in tropical regions, are mainly composed of calcareous material that are characterized by high permeability and high porosity (Schroeder et al., 1986; Rasheed et al., 2004). These sediments have several unique biogeochemical features. First of all, sandy reef sediments usually occur in physically active environments, which promotes rapid sediment-seawater exchange (Hunter et al., 2006). Secondly, because of their skeletal origin, reef carbonate sediments have a high specific surface area (due to the presence of small channels and crevices) and support relatively high numbers of microorganisms (Rasheed et al., 2003, 2004; Wild et al., 2004, 2006). Indeed, bacterial numbers in reef sands, ranging from 1.7×10^9 to 2.5×10^9 cell/cm³ (Wild et al., 2006), are one order of magnitude higher than that described for silicate sands of a similar grain size spectrum. Finally, frequent resuspension and deposition cycles in sandy sediments, resulting from strong physical forcing, winnow fine and low density materials from the sediment can cause microbes to adhere to sand grains or to move deeper into the sediment (van Raaphorst et al., 1998; Rusch et al., 2003). Thus, partitioning of microbial cells between sandy sediments and their pore fluids may depend on complex interactions between biotic and abiotic factors, including, but not limited to, the physical and chemical properties of the environment, the morphology and physiology of the microbial cells, and the hydrogeological properties of the sediments (Heise et al., 1999; Simoni et al., 2000; Shimeta et al., 2001).

The physical characteristics of sandy reef sediments makes conventional sediment sampling methods for microbial biogeochemical studies very difficult, if not impossible (Hannides, 2008). For example, it is hard to collect an intact sandy reef sediment core without significantly disturbing the established redox gradient because the presence of gravel-sized and larger biogenic clasts disrupts coring, and because the permeable nature of these

sediments leads to pore water loss during core recovery. Therefore, the study of the relationship between microbial communities and redox environments in reef sandy sediments poses significant challenges when using conventional sampling approaches.

We hypothesize that redox stratification of sandy reef sediments will stratify bacterial communities, and will result in observable microbial community changes across sedimentary redox gradients. To overcome the sampling obstacles mentioned above, we studied the distribution of bacterial communities in redox-stratified reef sandy sediments using a newly designed microcosm that mimics the functioning of natural permeable sediments. The main advantage of the microcosm is to allow uncontaminated samples to be collected from specific depths across the vertical redox gradient in the sediment, something that is not possible with conventional field-collected samples.

2 MATERIAL AND METHOD

2.1 Sample collection and microcosm operation

Sandy sediments used in this study were collected from the site of the Kilo Nalu Nearshore Reef Observatory, a cabled physical-biogeochemical ocean observing system along the south coast of Oahu, Hawaii (Sansone et al., 2008). Sediments for this study were collected from a 6 m×6 m sand field. Before use, sediments were transferred 2–3 times into separate buckets containing twice-filtered (0.2 μm) Waikiki Aquarium (Honolulu, Hawaii) seawater (FSW), to remove pore water and very fine suspended particulates from the sediments. These steps also served to homogenize the sediment, which prevented aggregated organic material from producing redox “hotspots”, and prevented the formation of channels in the microcosm, which could produce non-representative local pore water concentrations of nutrients. In addition, rock fragments greater than 15 mm in diameter were removed to minimize heterogeneous sediment packing during the loading of the microcosm, and to prevent interference with sample collection. The resulting sediment for this study was characterized as having the following properties: median grain size, 0.23 mm; mean grain size, 0.24 mm; sorting coefficient, 0.78; porosity, 0.51; permeability, 4.3×10^{-11} m².

These sediments were incorporated into a laboratory microcosm that mimicked the rapid sediment-seawater exchange observed in the field (Hannides, 2008). In brief, the microcosm (Fig.1)

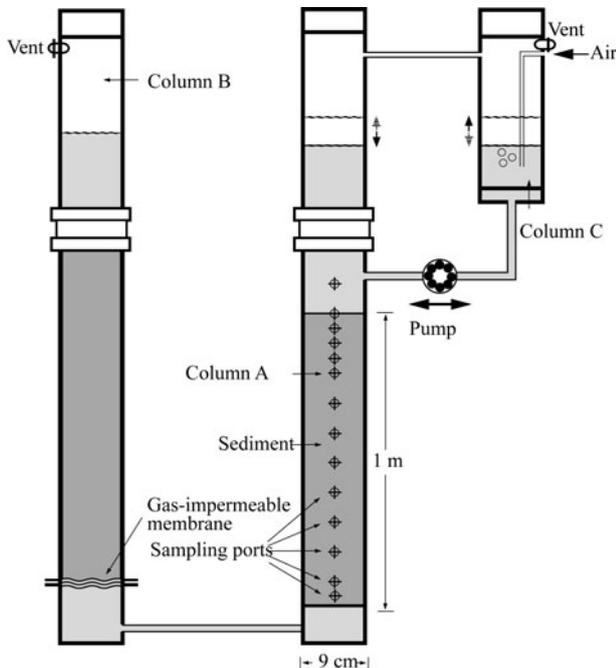


Fig.1 Schematic diagram of the microcosm used to study the rapid sediment-seawater exchange observed in the field

consisted of two connected columns (Column A and B), each approximately 2 m high. Column A, which was studded with sampling ports, contained the sediments under investigation. Column B was packed with combusted silica sand over a flexible, gas-impermeable membrane to prevent hydraulic and chemical interaction between the bottom of the sediment in Column A and the overlying water. Column C was a reservoir of FSW that was pumped in and out of the top of Column A to mimic the effect of surface waves on natural reef sediments, the process that dominates the exchange of pore water and seawater in the sandy reef sediments at Kilo Nalu (Hebert et al., 2007). To prevent contamination, the columns were sterilized with 75% ethanol prior to use and covered with aluminum foil during operation.

The microcosm was operated until it reached a steady state that mimicked the geochemical conditions of sediments in the field. The primary indicators of the state were the shape of the dissolved oxygen and inorganic nutrient profiles in the sediment (Hannides, 2008). Samples for nucleic acid extraction were collected after this point had been reached, 75 d after the microcosm was established, thereby allowing microbial characterization of well-defined geochemical regimes in the sediment.

Three ~1.5 g sediment samples from each of the oxic (0–1 cm below the sediment-water interface), suboxic (3–5 cm), and anoxic (15–25 cm) sediment

layers were collected and well mixed for microbial analyses. The concentrations of dissolved oxygen, inorganic nitrogen (DIN, defined as the sum of ammonium (the primary component), nitrate (NO_3^-) and nitrite (NO_2^-)), and dissolved inorganic phosphate (DIP), and the presence of sulfide in microcosm pore water were determined (Hannides, 2008).

2.2 DNA extraction, cloning and library analysis

Total genomic DNA was extracted from sediment samples using a FastDNA Kit (MP Biomedicals) according to the manufacturer's instructions. The resulting total genomic DNA was used as a PCR template for the amplification of 16S rRNA, *amoA*, and *nosZ* genes using the primer pairs 27f/1406r (Lane et al., 1988; Gurtler et al., 1996), nos752f/nos1773r (Scala et al., 1998; Hunter et al., 2006), and *amoA*-1f/*amoA*-2r (Rotthauwe et al., 1997; Hunter et al., 2006), respectively. A 16S rRNA library was constructed using a method described previously (Zhu et al., 2008). Positive clones were grouped based on restriction fragment length polymorphisms (RFLP) patterns using the restriction enzymes *MspI* and *RsaI* (Promega, Madison, WI). One representative clone from each group was cultured for the plasmid DNA isolation and sequencing analysis.

2.3 Denaturing gradient gel electrophoresis (DGGE) analysis

All PCRs for DGGE analysis were performed using the Expand High Fidelity PCR System (Roche) according to the manufacturer's instructions. The total genomic DNA extracted from the sediment samples was used as a PCR template for the amplification of the 16S rRNA gene using the primers 341f-GC and 907r, as described by Shafer and Muyzer (2001). Denaturing gradient gel electrophoresis analysis was performed using a model DGGE-2001 electrophoresis system (C.B.S Scientific Company Inc.) with a denaturing gradient of 30%–70% in a 7.5% polyacrylamide gel, with bands cloned for sequencing analysis, as described previously (Gao et al., 2008).

2.4 Sequence and phylogenetic analysis

Plasmids were sequenced at the University of Hawaii DNA Core Sequencing Facility on an Applied Biosystems 3730XL automated DNA sequencer. Sequence data were edited with the Chromas Lite, Version 2 (Technelysium) software package. Chimeric sequences were checked using the Ribosomal Database Project (RDP) II Chimera Check program (<http://rdp8.cme.msu.edu/cgi/>

chimera.cgi). Clone sequences were grouped using DOTUR (Schloss and Handelsman, 2005). The 16S rRNA gene sequences and *AmoA* and *NosZ* protein sequences were derived from *amoA* and *nosZ* genes, respectively, from each library with a percent sequence identity of greater than 97% being placed in the same OTU (Operational Taxonomic Unit). One sequence from each OTU was selected as a representative for further analysis. For preliminary identification, sequences of bacterial 16S rRNA genes and *AmoA* and *NosZ* proteins were compared with those deposited in the NCBI database (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>) and 16S rRNA gene sequences were additionally compared with those in Ribosome Database Project (RDP) using Classifier (<http://rdp.cme.msu.edu/classifier/classifier.jsp>). The representatives of bacterial 16S rRNA sequences in this study and the matched sequences from GenBank were aligned using BioEdit, version 7.0.5.3 (Hall, 1999). Phylogenetic trees were constructed using the neighbour-joining method implemented in PAUP* 4.0b10 described by Zhu et al. (2008). The quality of the branching patterns for the NJ trees was assessed by bootstrap resampling of the data sets with 1 000 replications. The percentage coverage (C) of the clone libraries was calculated according to the following equation: $C = [1 - (n_i/N)] \times 100$ (Good, 1953; Mullins et al., 1995), where n_i is the number of unique clones, and N is the total number of clones in the library. The diversity within the libraries was measured with the Shannon-Weaver index (H) and evenness (E) using the equation $H = -\sum P_i \ln(P_i)$, where P_i is the proportion of the total number of OTUs made up to the i^{th} OTUs, and $E = H/\log(S)$, where S is the total number of OTUs in the community. Comparisons of the clone libraries were made using the J-LIBSHUFF program (<http://www.plantpath.wisc.edu/fac/joh/S-libshuff/downloads.html>) (Accessed on 2011-9-1) (Schloss et al., 2004).

2.5 Nucleotide sequence accession number

Bacterial 16S rRNA gene sequences obtained in this study were deposited in GenBank under accession Nos. FJ358843–FJ358930. The *amoA* and *nosZ* gene sequences were also deposited in GenBank under accession Nos. FJ969044–FJ969112.

3 RESULT

3.1 Pore water geochemistry

Pore water dissolved oxygen and nutrient concentrations (i.e. DIN and DIP) were measured

Table 1 Pore water geochemical characteristics of the sandy reef sediments in the microcosm columns

Layer	Depth (mm)	Dissolved oxygen ($\mu\text{mol/L}$)	Sulfide	DIN ($\mu\text{mol/L}$)	DIP ($\mu\text{mol/L}$)
Oxic	0–10	185–207	Not detected	<0.31–0.38	0.17–0.43
Suboxic	30–50	<5–14	Not detected	9.0–30.0	2.8–3.2
Anoxic	150–250	<5	Faint to definite	240–360	6.3–7.8

Data were collected 3.5 d before the end of the 75-d study

in the three different redox zones in the upper sediments of the microcosm experiments (Table 1). The dissolved oxygen content showed a large concentration gradient from the oxic to anoxic layers, with levels <5 $\mu\text{mol/L}$ in the anoxic pore water. Large concentration gradients were also observed for DIN and DIP, with their highest concentrations being detected in the anoxic pore water. The steep decrease in dissolved oxygen and increase in the inorganic nutrient concentration from the oxic to anoxic layer suggest rapid microbial mineralization of organic matter in these sandy reef sediments. However, only a minimum amount of sulfide was detected in the anoxic pore water, as is common in such sediments (Sansone et al., 1990). In addition, the contribution of nitrate and nitrite to the total DIN concentrations (data not shown) was insignificant, presumably reflecting the low nitrification and/or high denitrification activity present.

3.2 Bacterial diversity analysis based on 16S rRNA genes

The bacterial 16S rRNA gene-DGGE band patterns varied in the oxic, suboxic, and anoxic sandy sediments (Fig.2a–b). A total of 15, 12, and 18 bands were detected from the DGGE profiles in the three zones, respectively. The high number of bands detected in the DGGE analysis suggested a large bacterial diversity in these sediments, with the greatest bacterial diversity found in the anoxic layer. Nine bands were present in all three layers. One band detected in the oxic sediment layer was absent in both the suboxic and anoxic sediment layers, and five bands present in the anoxic layer were absent in both the oxic and suboxic layers. These findings suggested that the three redox sandy sediment layers contained many of the same bacterial phylotypes, but both the oxic and anoxic sediments contained unique bacterial phylotypes.

Separated 16S rRNA gene fragments in the DGGE gels were excised and sequenced to identify bacterial

phylotypes in the three zones. A total of 22 bands were excised from the DGGE gel and used for the construction of the 16S rRNA gene libraries. A total of 192 clones were sequenced, out of which 11 yielded chimeric sequences that were excluded from further analyses. Group analyses identified 11, 9, and 13 OTUs for the oxic, suboxic, and anoxic sandy sediments, respectively. These OTUs are the representatives of the phyla *Proteobacteria* (classes *Alpha-*, *Beta-*, and *Gamma-proteobacteria*), *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, and *Firmicutes*. The highest diversity of bacterial communities was found in the anoxic sediment layer and the least in the suboxic layer.

The strength of the DGGE analysis as a microbial fingerprinting method is to provide a rapid method to study and compare the community structure in response to changes in environmental parameters over space and time (Nakatsu, 2007). Furthermore, DNA fragments in the individual DGGE bands can be cloned and sequenced for the identification of microbial population changes associated with community changes or differences. However, this method, because of its weaknesses such as gel-to-gel variation, limited phylogenetic information, and underestimate of overall diversity, is just a good starting point for more in-depth studies. To have a complete review of the structure and composition of bacterial communities in the different redox environments, three clone libraries were constructed from each of the redox sediment layers. The diversity indices of the three 16S rRNA clone libraries are summarized in Table 2. Overall, 19, 12, and 24 OTUs were detected from the three clone libraries

constructed from the oxic, suboxic, and anoxic sediment layers, respectively.

Regarding the diversity and distribution of the microbial communities in the different redox layers, the results of the 16S clone libraries were consistent with those of DGGE band patterns and DGGE clone libraries, with the greatest and the least bacterial community diversity found in the anoxic and suboxic layers, respectively (Table 1). However, many more bacterial groups were detected in the 16S clone libraries than in the DGGE clone libraries for each redox layer. Two phyla (*Verrucomicrobia* and *Spirochaetes*), three candidate divisions (WS3, SO31, and AO19), and cyanobacteria-like sequences were only present in the 16S clone libraries, while two phyla (*Chloroflexi* and *Firmicutes*) were only found in the DGGE clone libraries. In addition, two classes of the phylum *Proteobacteria* (*Delta-* and *Epsilon-proteobacteria*) were found only in the 16S clone libraries. Overall, the bacterial phylotypes resulting from this study were members of nine phyla and three candidate divisions. In addition, a bacterial phylotype of the WS3 candidate division is being reported here for the first time, for permeable sediments.

3.3 Phylogenetic analysis based on the 16S rRNA genes

Sequence analysis of 88 OTUs derived from 3 clone libraries (55 OTUs) and from the DGGE analysis (33 OTUs) revealed 9 distinct phyla and 3 candidate divisions, with the majority of the OTUs mostly being closely related to clone sequences previously described from marine sediments. Still, some of the OTUs were related to sequences derived

Table 2 Statistical analyses of the 16S rRNA, *amoA*, and *nosZ* clone libraries constructed from the oxic, suboxic, and anoxic layers of the microcosm

PCR target	Sample	No. of clones (No. phylotypes)	Coverage ^a (%)	Shannon-Weaver index (H^b)	Species richness (S^c)	Evenness (E^c)
16S rRNA gene	Oxic	78 (19)	84	2.6	19	0.88
	Suboxic	73 (12)	92	1.75	12	0.70
	Anoxic	73 (24)	77	1.90	24	0.60
<i>nosZ</i>	Oxic	41 (17)	81	2.47	17	0.87
	Suboxic	33 (16)	70	2.47	16	0.89
	Anoxic	38 (20)	68	2.66	20	0.89
<i>amoA</i>	Oxic	24 (7)	79	1.1	7	0.57
	Suboxic	23 (3)	96	0.77	3	0.70
	Anoxic	15 (2)	100	0.4	2	0.58

^a Coverage = $1 - (n_i/N) \times 100$, where n_i is the number of OTU's appearing only once in the library and N is the total number of clones examined

^b $H = -\sum P_i \ln(P_i)$

^c $E = H/\ln(S)$

^d S = total number of OTUs in the community

from seawater, corals and terrestrial habitats, including plants, animals, insects, and soils. The phylogenetic relationships for the OTUs derived from this study, with reference sequences from GenBank, are presented in Fig.2a–d. Some of these OTUs are potentially new bacterial phylotypes because they had low sequence similarity with sequences deposited in GenBank and did not have an immediate phylogenetic neighbour (e.g., OXD4-09).

Members of *Proteobacteria* identified were representatives of *Alpha*-, *Beta*-, *Delta*-, *Epsilon*-, and *Gamma*-*proteobacteria* (Fig.2a–b) and

constituted 44% of all bacterial phylotypes. Alphaproteobacterial OTUs (Fig.2a) were found in all three redox layers and were closely related to bacterial phylotypes derived from seawater, deep-sea sediments, marine sponges, and corals. Of the betaproteobacterial phylotypes, three OTUs (OXD4-12, OX39 and OXD7-11) were found in the oxic sediment layer and one (SOD6-1) was found in the suboxic layer. Gammaproteobacterial phylotypes were branched into two clusters, with four members of clade A found in all three redox layers (Fig.2a). Deltaproteobacterial OTUs were the singularly most abundant bacterial phylotypes detected in this study.

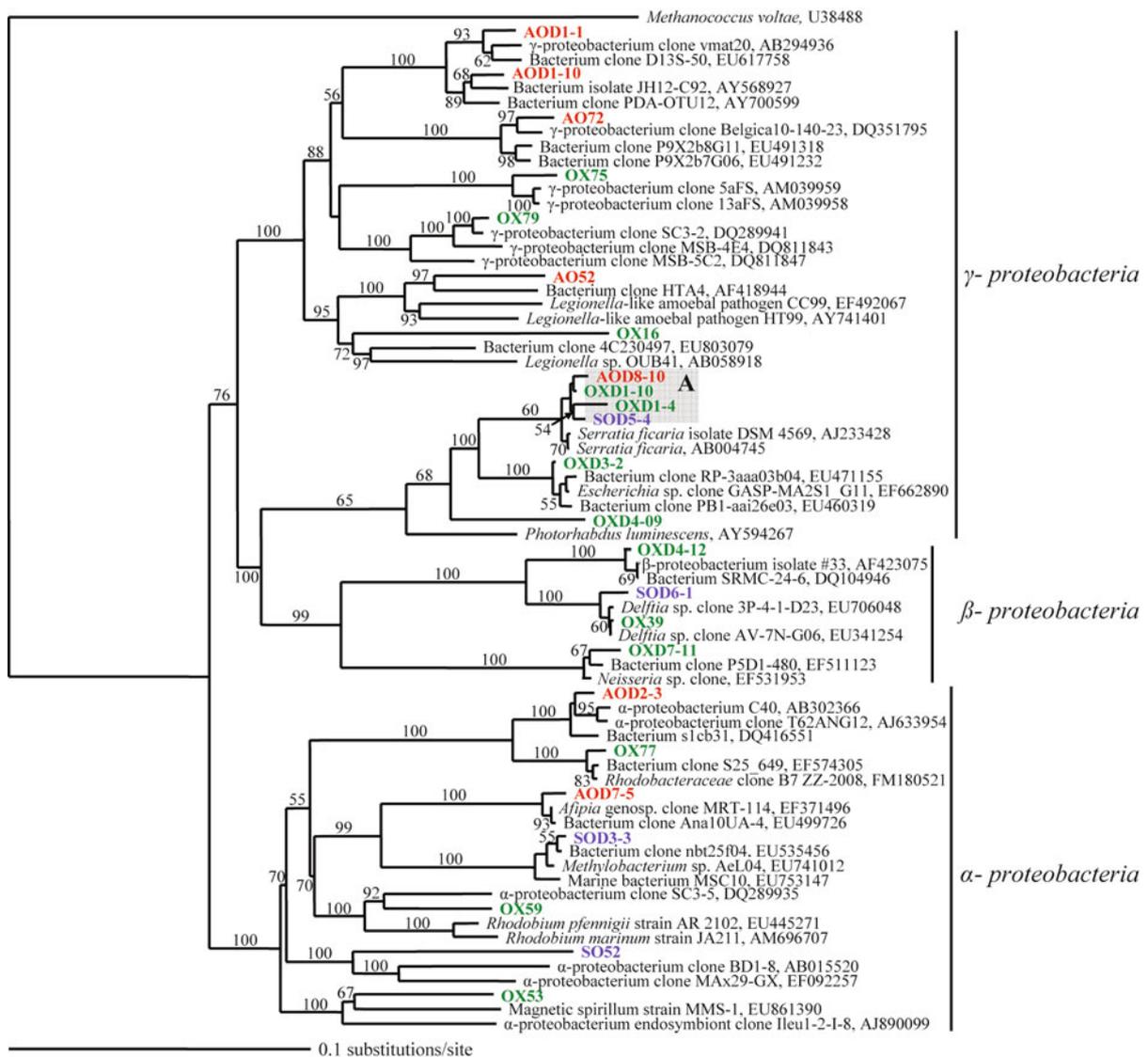


Fig.2a Phylogenetic trees based on 16S rRNA gene sequences related to *Alpha*-, *Beta*-, and *Gamma*-*proteobacteria* a. *Epsilon*- and *Delta*-*proteobacteria*; b. *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Firmicutes*; c. and *Acidobacteria*, *Spirochaetes*, *Verrucomicrobia*, *Cyanobacteria* relatives and other candidate divisions; d. Clone sequences from the 16S rRNA libraries of the oxic, suboxic, and anoxic sandy layers are designated with the prefix OX, SO, and AO, respectively (in bold). Clone sequences from DGGE band clone libraries of the oxic, suboxic, and anoxic layers are designated with the prefix OXD, SOD, and AOD, respectively (in bold). Clone sequences derived from the oxic, suboxic, and anoxic sediment layers are in green, purple, and red, respectively. Shaded clades (A, B, C, D, and E) include OTUs from all three sediment layers.

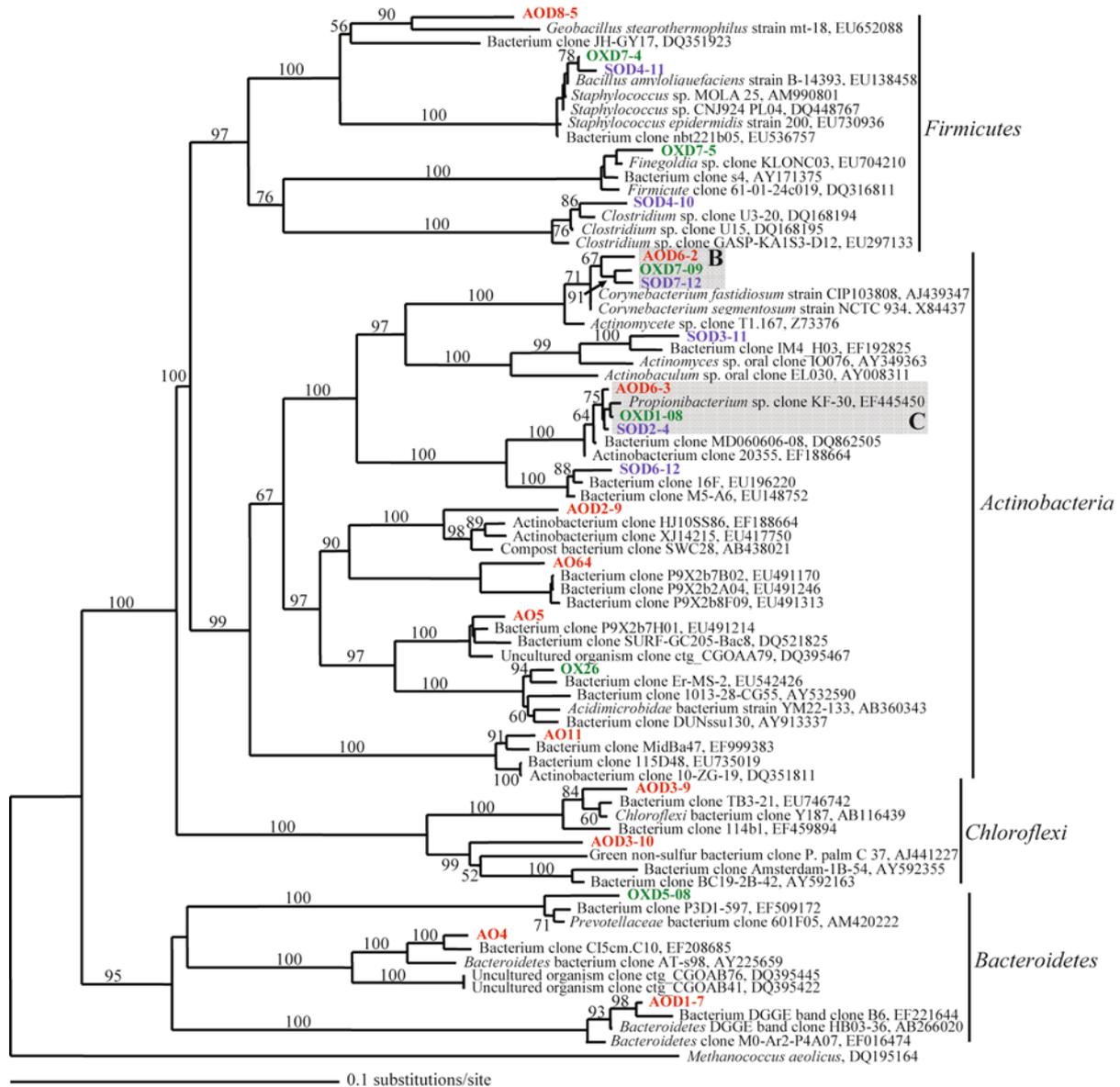


Fig.2c Phylogenetic trees based on 16S rRNA gene sequences related to *Alpha*-, *Beta*-, and *Gammaproteobacteria*

a. *Epsilon*- and *Delta*-*proteobacteria*; b. *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Firmicutes*; c. and *Acidobacteria*, *Spirochaetes*, *Verrucomicrobia*, *Cyanobacteria* relatives and other candidate divisions; d. Clone sequences from the 16S rRNA libraries of the oxic, suboxic, and anoxic sandy layers are designated with the prefix OX, SO, and AO, respectively (in bold). Clone sequences from DGGE band clone libraries of the oxic, suboxic, and anoxic layers are designated with the prefix OXD, SOD, and AOD, respectively (in bold). Clone sequences derived from the oxic, suboxic, and anoxic sediment layers are in green, purple, and red, respectively. Shaded clades (A, B, C, D, and E) include OTUs from all three sediment layers.

be present only in the anoxic portion of sediments from the Antarctic continental shelf (Bowman and McCuaig, 2003). Results of the present study suggest that Acidobacterial phylotypes may exhibit a similar zonation in sandy reef sediments as well.

Cyanobacterial OTUs were identified from all three redox layers and branched into three distinct groups (Fig.2d). Three OTUs (AO26, AO62 and SO78) of the first group, from the anoxic and suboxic layers, were closely related to cyanobacterial clone sequences from permeable sediments from the South

Atlantic Bight (SAB) (Hunter et al., 2006), a shallow submarine hydrothermal system (Hirayama et al., 2007), and a coral reef sediment (Sørensen et al., 2007), as well as *Xenococcus* sp. sequences (Turner et al., 1999). Ten OTUs formed the second group and were members of plastids from marine algae or diatoms (Fig.2d). Four OTUs (AO1, SO47, OX49, and AO58) branched into a distinct clade (D) and were found throughout the three redox environments. In comparison with previous work (Hunter et al., 2006; Sørensen et al., 2007; Mills et al., 2008), we

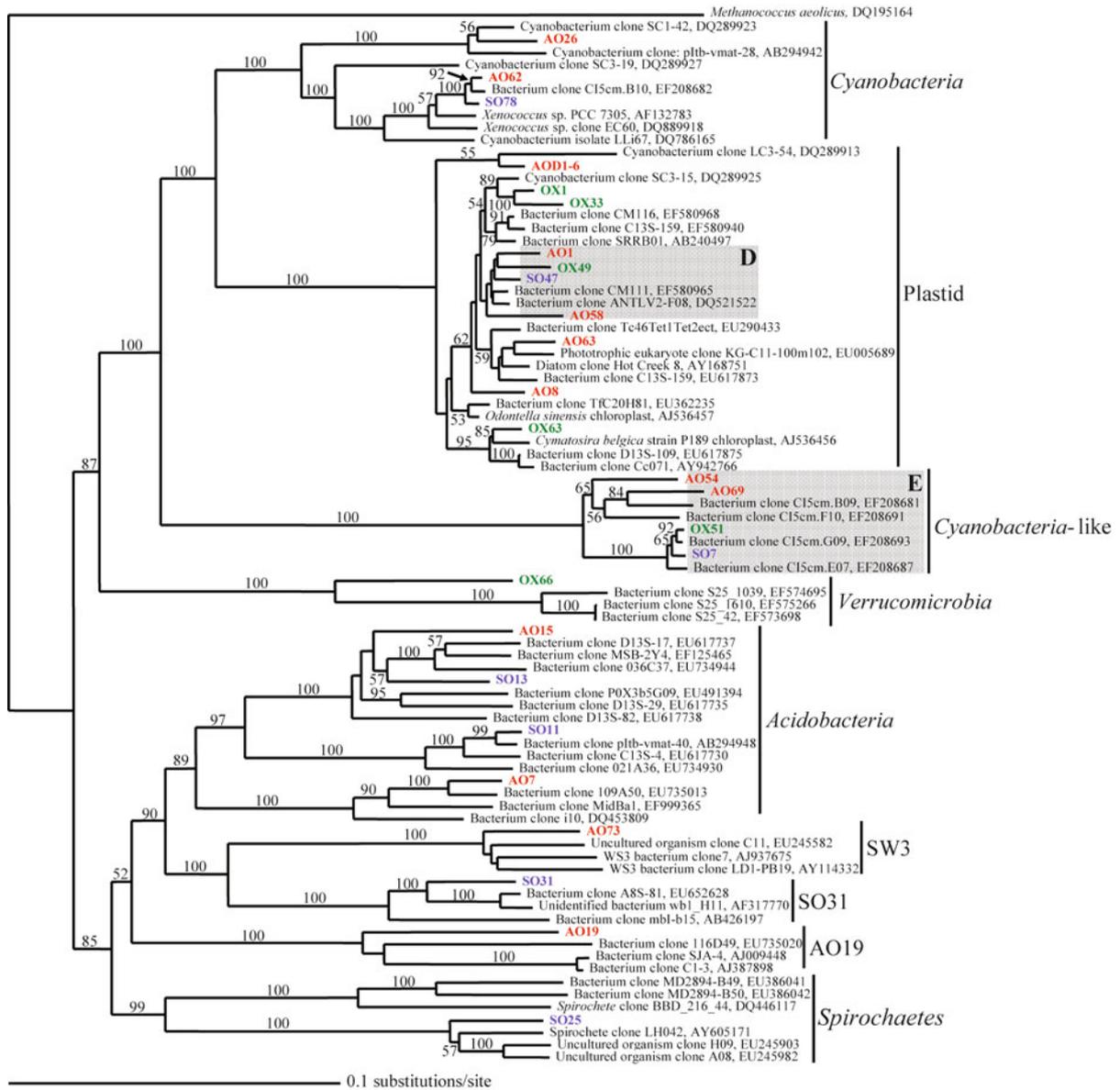


Fig.2d Phylogenetic trees based on 16S rRNA gene sequences related to Alpha-, Beta-, and Gammaproteobacteria

a. Epsilon- and Delta-proteobacteria; b. Actinobacteria, Bacteroidetes, Chloroflexi, and Firmicutes; c. and Acidobacteria, Spirochaetes, Verrucomicrobia, Cyanobacteria relatives and other candidate divisions; d. Clone sequences from the 16S rRNA libraries of the oxic, suboxic, and anoxic sandy layers are designated with the prefix OX, SO, and AO, respectively (in bold). Clone sequences from DGGE band clone libraries of the oxic, suboxic, and anoxic layers are designated with the prefix OXD, SOD, and AOD, respectively (in bold). Clone sequences derived from the oxic, suboxic, and anoxic sediment layers are in green, purple, and red, respectively. Shaded clades (A, B, C, D, and E) include OTUs from all three sediment layers.

report a very diverse set of plastid sequences from our marine sediments. Finally, four OTUs (AO54, AO69, SO7, and OX51) clustered into a distinct group with *Cyanobacteria*-like sequences from coral reef sediments were collected around the island of Oahu, Hawaii (Sørensen et al., 2007), and were found in all three redox environments (Fig.2d). Because of their low sequence identity (79%) with those in GenBank, they are likely to be derived from a new group of free-living bacteria or a new group of eukaryotes.

The OTU SO25 from *Spirochaetes* was identified from the suboxic layer and branched into a group with clone sequences from a marine microbial mat (Berlanga et al., 2003; Isenbarger et al., 2008). The OTU AO73 from the anoxic layer formed a distinct group similar to WS3 clone sequences from anaerobic marine sediments collected from Baltimore harbour (Nesbo et al., 2005) and Loch Duich (Scotland) (Freitag et al., 2003), as well as a clone sequence from a hypersaline microbial mat (Isenbarger et al., 2008). The OTU SO31 branched into a group with

a clone sequence from the Yellow Sea sediment and a novel lineage sequence from the microbial mats of the Nullarbor caves in Australia (Holmes et al., 2001). Because of the low sequence similarity with reference sequences, it was placed in the candidate division SO31. The OTU OX66 was distantly (<93%) related to the *Verrucomicrobial* clone sequences from seawater, and is potentially a new genus (Fig.2d). Finally, the OTU AO19 was distantly (<87%) related to unclassified bacterial sequences from an anaerobic trichlorobenzene-transforming microbial consortium (von Wintzingerode et al., 1999, 2000), and those from a sediment from the northern Bering Sea, and was placed into candidate division AO19 (Fig.2d).

3.4 Comparison of 16S rRNA clone libraries

A comparison of 16S rRNA clone libraries constructed from the three oxic, suboxic, and anoxic sediment layers was calculated using the β -LIBSHUFF program (Schloss et al., 2004). Using the integral form of the statistics imbedded in β -LIBSHUFF with 10 000 randomizations, we found that all the comparisons were highly significant ($P < 0.001$, Table 3). This result suggests that there is a high probability that the 16S rRNA gene libraries constructed from the oxic, suboxic, and anoxic layers contained different taxonomic lineages. To determine whether this was a reasonable outcome, we compared the taxonomic distribution of the three libraries (Table 3). There were large differences between them in the relative abundance of the phyla presented in the redox-stratified sediments. Results of library comparison analyses support the conclusion that the clone libraries constructed from the three different redox environments contain different taxonomic lineages.

3.5 Diversity and phylogenetic analysis based on the *amoA* genes

Nitrification is the microbial oxidation of ammonium (NH_4^+) to nitrate via nitrite (Beman et al., 2006). Its first step, oxidation of NH_4^+ to NO_2^- , is catalyzed by ammonia monooxygenase (Amo), which is considered the rate-limiting step of nitrification in ammonia-oxidizing bacteria (AOB). The *amoA* gene, which encodes for a subunit of the Amo enzyme, has been used as a marker gene to investigate AOB communities in a variety of natural habitats, including marine environments (O'Mullan et al., 2005; Beman et al., 2006; Hunter et al., 2006; Mills et al., 2008; Sahan et al., 2008; Urakawa et al., 2008). Sequence analysis of three *amoA* clone

Table 3 A comparison of the 16S rRNA, *amoA*, and *nosZ* libraries^a for the three redox environments

Library source	Homologous library (X)	P value of ΔC_{xy} heterologous library (Y)		
		Oxic	Suboxic	Anoxic
16S rRNA	Oxic		0.000 8	0.000 9
	Suboxic	0.000 5		0.000 3
	Anoxic	0.001 3	0.000 24	
<i>amoA</i>	Oxic		0.115 3	0.000 2
	Suboxic	0.699 4		0.000 0
	Anoxic	0.069 7	0.012 3	
<i>nosZ</i>	Oxic		0.001 0	0.000 1
	Suboxic	0.006 1		0.045 8
	Anoxic	0.071 2	0.138 2	

Comparisons were made using β -LIBSHUFF to calculate the integral form of the Cramér-von Mises statistic under 10 000 randomizations. The margins of error for the 95% confidence intervals for the three layers were 0.004, 0.000 0, and 0.0002 for 16S rRNA, *amoA*, and *nosZ* libraries, respectively.

ΔC_{xy} = the coverage difference of two clone libraries (x and y).

libraries identified 7, 3, and 2 OTUs in the oxic, suboxic, and anoxic sediment layers, respectively (Table 2). Using the β -LIBSHUFF program (Schloss et al., 2004), a comparison of *amoA* clone libraries constructed from the three layers yielded significant differences for the clone library pairs from the oxic/anoxic and suboxic/anoxic layers ($P < 0.002$, Table 3). This result suggests that there is a high probability that AOB phylotypes in the oxic sediment and suboxic sediment layers are different from those in the anoxic sediment layer.

Phylogenetic analysis indicated that all *amoA* sequences derived from the sediments are exclusively related to those described from marine environments and belong to members of the betaproteobacterial AOB phylotypes. These sequences were clustered into four clades (F, G, H, and I) (Fig.3). Members of clade F and H were present in all three redox layers. Members of clade G were only present in oxic sediment layers. Members of clade I were found in both the oxic and suboxic sediment layers and are likely new phylotypes because they do not have immediate phylogenetic neighbours. No AOB OTUs were present only in the anoxic sediment layer. The *amoA* gene analysis results of this study are consistent with the previous reports that the diversity of bacterial nitrifiers (*amoA*) is low in permeable marine sediments (Hunter et al., 2006; Mills et al., 2008). Overall, redox-stratification seems to influence the diversity and distribution of nitrifiers in sandy reef sediments, with more nitrifiers present in the oxic layer than the other layers.

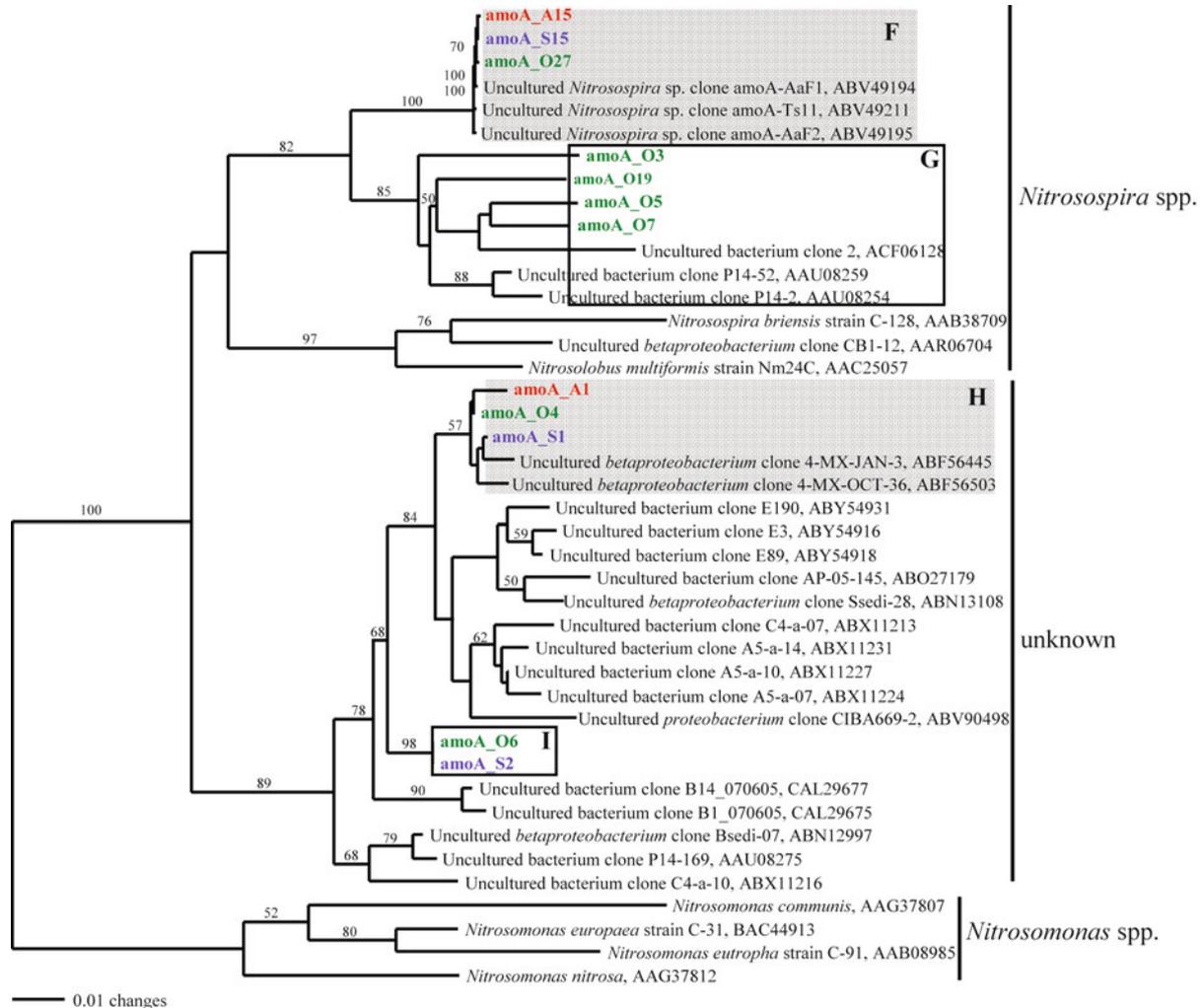


Fig.3 Distance neighbor-joining tree of the betaproteobacterial AmoA sequences based on 160 amino acid residues
 Bootstrap values of >50 are displayed. Clone sequences derived from oxic, suboxic, and anoxic sediment layers are in green, purple, and red, respectively. Shaded clades (F and H) include OTUs from all three sediment layers.

3.6 Diversity and phylogenetic analysis based on the *nosZ* genes

Denitrification is the reduction of nitrate or nitrite to nitrogenous gases, which generally occurs under anaerobic conditions, and is an important part of marine nitrogen cycling (Horn et al., 2006; Brandes et al., 2007). Many genes involved in microbial denitrification have been identified and studied in detail (Philippot, 2002 for a review). These genes include *narGHI* (encodes nitrate reductase), *nirK* or *nirS* (encodes nitrite reductase), *norBC* (encodes NO reductase), and *nosZ* (encodes N₂O reductase) (Zumft, 1997). Nitrous oxide reductase encoded by the *nosZ* gene is associated with the last step of the denitrification process and this gene has been commonly used as a marker for the molecular analysis of the majority of denitrifiers in marine sediments (Scala et al., 1998, 1999, 2000; Nogales et al., 2002; Horn et al., 2006; Hunter et al., 2006).

Sequence analysis of the three *nosZ* gene clone libraries identified 17, 16, and 20 OTUs in the oxic, suboxic, and anoxic sediments, respectively (Table 2). The comparative analysis of the three *nosZ* clone libraries derived from these layers using the β -LIBSHUFF program (Schloss et al., 2004) indicated that there were significant differences in denitrifier populations in the three layers presumably reflecting the different redox conditions present ($P < 0.045$) (Table 3).

Phylogenetic analysis indicated that *nosZ* sequences derived from this study were largely affiliated with those derived from other permeable sandy sediments (Fig.4). Some of these *nosZ* sequences were found in all three redox sediment layers ($n=22$, 42%), and others ($n=24$, 45%) were present in two of the three sediment redox layers. However, others ($n=7$, 13%) were only present in one of the layers. Additionally, nine OTUs (e.g.,

nosZ_S3p and nosZ_S9p) were potential new phylotypes because they did not have immediate phylogenetic neighbors. In contrast to the nitrifiers, an analysis of *nosZ* genes suggested that there was indeed a much greater diversity of denitrifiers present in all three sediment layers.

4 DISCUSSION

Bacterial communities play a fundamentally important role in the mineralization of organic matter

through diverse geochemical transformations occurring in marine sediments (Jorgensen et al., 2007). Microbial mineralization is largely regulated by the redox environment (or, similarly, dissolved oxygen concentration) in the pore water, which, in turn, is directly influenced by microbial metabolic activities, and vice versa. Thus, based on the levels of dissolved oxygen, marine sediments have been commonly characterized by three zones: “oxic”, “suboxic”, and “anoxic” (Burdige, 2006). In other words, mineralization in marine sediments occurs in

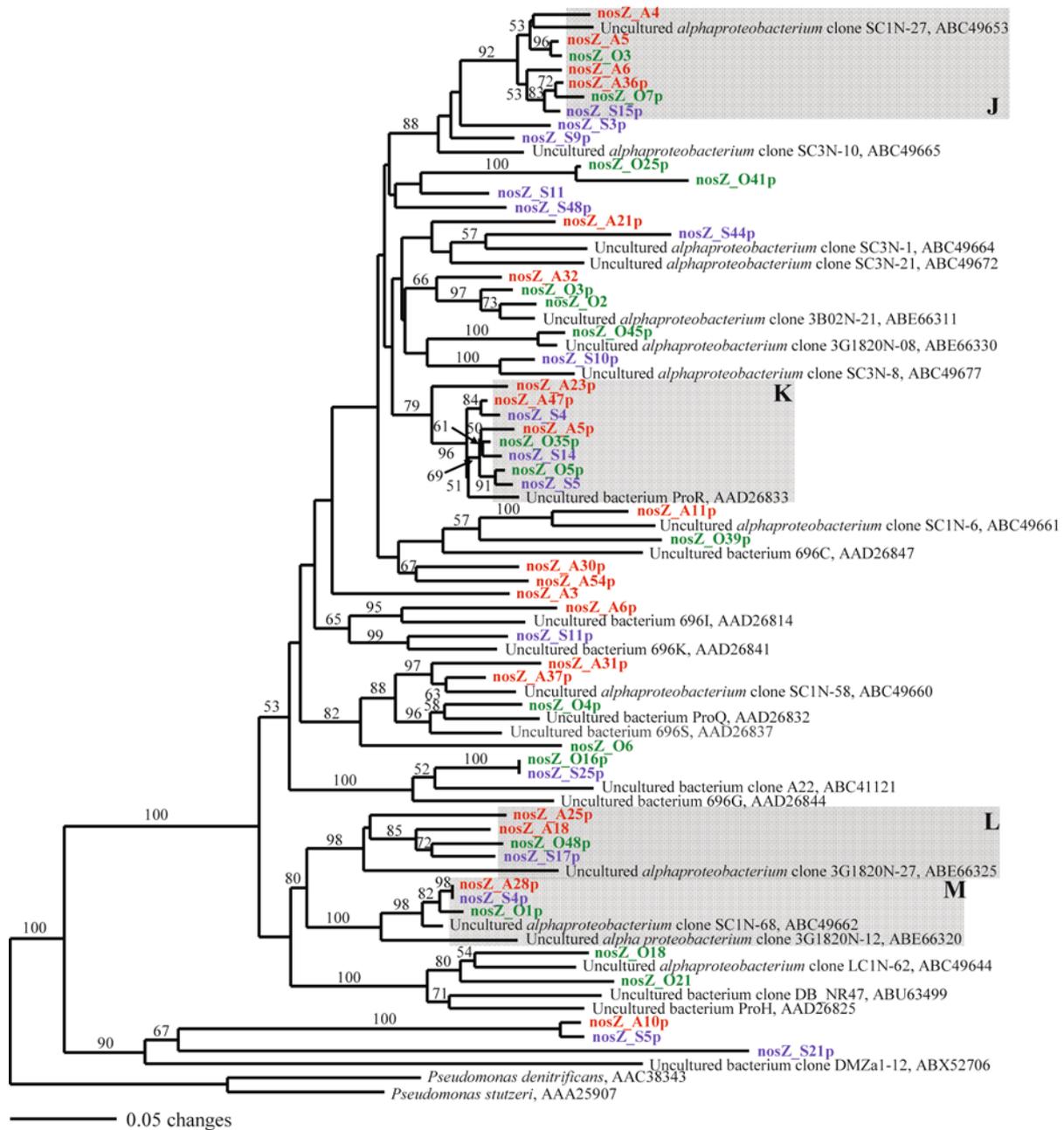


Fig.4 Distance neighbor-joining tree of the alphaproteobacterial *NosZ* sequences based on 310 amino acid residues
 Bootstrap values of >50 are displayed. Clone sequences derived from oxic, suboxic, and anoxic sediment layers are in green, blue, and red, respectively. Shaded clades (J, K, L, and M) include OTUs from all three sediment layers.

biogeochemical and mineralogical zones that may reflect the dominant microbial communities growing at a particular depth, although in some cases these communities may overlap.

This study revealed diverse bacterial phylotypes, including novel ones, as well as bacterial stratification and zonation in the redox-stratified sediments in the microcosm, as was shown in the DGGE and clone library analyses (Fig.2a–d, Fig.1 and Table 1). We conclude that redox-stratification enhances bacterial stratification and zonation, as evidenced by the fact that the sediments from the three different redox environments in the microcosm were from the same site and were well mixed prior to being loaded into the microcosm. Thus, microbial communities in the three sediment layers were presumably similar, if not identical, at the beginning of the microcosm experiment. Therefore, the difference of bacterial phylotypes in the individual redox layers should directly result from the redox stratification in the microcosm. Although evidence for the existence of distinct biogeochemical zones in the sediments has been known for more than half a century, a depth-related scheme describing in detail the microbial communities in each layer has not yet been developed. This study provides the first data on the vertical structure of the microbial community in redox-stratified sandy reef sediments.

The vast majority of bacterial phylotypes identified in this study are closely related to clone sequences previously identified in other marine sediments (Llobet-Brossa et al., 1998, 2002; Bowman et al., 2003; Buhring et al., 2005; Hunter et al., 2006; Musat et al., 2006; Sørensen et al., 2007; Edlund et al., 2008; Mills et al., 2008; Zhang et al., 2008). However, in contrast to previous studies on marine sediments, we observed that bacterial phylotypes of *Delta*- (16%) and *Gammaproteobacteria* (15%) and *Actinobacteria* (14%) appear to be the predominant bacterial phylotypes. Nevertheless, *Delta*- and *Gammaproteobacteria* were observed in deep-sea sediments from the northeastern Pacific Ocean (Xu et al., 2008). Additionally, bacterial phylotypes of *Planctomycetes*, which are commonly found in marine sediments and seawater, were not detected in the clone libraries of this study. In contrast, a relatively high percentage of plastid sequences was detected in the clone libraries, likely resulting from the high abundance of eukaryotic phytoplankton in Hawaiian waters.

Our results clearly indicate that the distribution of bacterial phylotypes displayed a correlation with redox stratification. For example, phylotypes of *Verrucomicrobia* were only found in the oxic

layer, while phylotypes of *Epsilonproteobacteria*, *Chloroflexi*, WS3, and AO19 were present only in the anoxic sediment (Table 1). Moreover, phylotypes of *Acidobacteria* were present in both the suboxic and anoxic sediments, whereas *Alpha*- and *Delta*-*proteobacteria*, *cyanobacteria-like*, *Firmicutes*, and *Actinobacteria* were detected in all three redox environments. Furthermore, comparison of *nosZ* and *amoA* gene libraries suggested that different denitrifier and nitrifiers occurred in three different redox layers (Tables 2 and 3).

Finally, most of the nitrifiers and denitrifiers we detected were closely related to phylotypes derived from marine sediments (Figs.3 and 4). However, some bacterial OTUs are likely novel phylotypes for nitrogen metabolizers because they did not have any close phylogenetic neighbor with sequences deposited in GenBank. Along with the novel cyanobacteria-like 16S rRNA sequences obtained in this study (Fig.2d) and in other reports (Sørensen et al., 2007), our results suggested that some bacterial phylotypes of reef sediments are biogeography- or sediment-specific.

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