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Succession of bacterial community structure and potential significance along a sediment core from site U1433 of IODP expedition 349, South China Sea

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ABSTRACT

To evaluate the potential impact of geological processes and depositional history on shaping the subsurface biosphere, the bacterial community structures in a sediment core of the South China Sea was investigated by molecular approaches that target 16S rRNA gene fragments. Samples were obtained from different lithologic intervals at site U1433 during the International Ocean Discovery Program (IODP) Expedition 349. Bacterial abundance decreased rapidly with depth, with nearly three orders of magnitude decline within the first 100 m below seafloor (mbsf). Community diversity displayed a similar decreasing pattern, yet, a slight increase in diversity emerged in the early to middle Miocene. Such excursion might reflect enhanced cell activity in response to increasing temperature due to a steep geothermal gradient. Non-metric multidimensional scaling ordination revealed that the bacterial communities along the sediment core represent four clusters based on depth and geological time. There were distinct bacterial community shifts among clusters at 4.50–98.93 mbsf (Pleistocene), 108.15–273.20 mbsf (Pleistocene), 296.09–709.13 mbsf (Pliocene and late Miocene), and 732.10–789.91 mbsf (early to middle Miocene). Classification analysis revealed a striking pattern: the relative abundance of microorganisms affiliated with *Gammaproteobacteria*, *Actinobacteria*, and *Cyanobacteria* overall consistently decreased with depth, whereas those affiliated with *Chloroflexi*, candidate division OP9, candidate phylum BHI80-139, and *Nitrospirae* increased; these findings correspond to different clusters. Total organic carbon content and ratio of total organic carbon: total nitrogen, along with pore water phosphate concentration and salinity, were the statistically most significant variables that explained the bacterial community cluster pattern, which indicates potential linkages of bacterial communities to changes in quality and quantity of buried organic matter over geological time scales. Geographic isolation across depth was more important than environmental condition and geological age for the development of unique community structure in marine deep biosphere, although environmental variables partially shaped bacterial community composition.

1. Introduction

The deep biosphere far below the seafloor is a central part of earth system processes over geological time scales, which may be sustained by the biosphere:geosphere interactions (Parkes et al., 2014). The deep biosphere harbors a vast ecosystem of bacteria, archaea, and fungi, with some unique biodiversity, and functions on geological time scales. Despite a clear common sharp decline in abundance with depth, cell-depth distributions vary in different oceanographic provinces, from organic-rich shelves/margin sediments to organic-poor oceanic gyre sediments. Most microbes have been shown to be active (Schippers et al., 2005; Biddle et al., 2006; Schippers et al., 2010; Lloyd et al., 2013) depending on very limited energy flux coupling with geological time scales (Hoehler and Jorgensen, 2013), and are therefore biogeochemically important.

Microbial communities usually change in response to surrounding geochemical conditions. Sediment receives deposition of microbes and organic matter from upper water layers, forming the largest reservoir of organic carbon (approximately $15{,}000 \times 10^{18}$ g, Hedges and Keil, 1995), and provides a matrix of complex nutrients and solid surfaces for microbial growth (Wang et al., 2012). On the other hand, sedimentary microbial communities may contribute to the degradation/alteration of organic matter and biogeochemical cycling as feedback. Although it is thought that intense degradation of sedimenting organic matter in the

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water column and near surface sediments has resulted in recalcitrant organic matter in sub-surface sediments (Jiao et al., 2010, 2014), we hypothesized that sediment microbial degradation changed further type and quantity of buried organic matter over geological time scales. Besides organic matter, the structure and function of these communities may be impacted by degree of oxygenation, nutrient levels, pH, and other factors across depth (Lüdemann et al., 2000; Fierer et al., 2003; Zhou et al., 2004; Hansel et al., 2008; Fierer and Jackson, 2006). To understand the potential effects of geochemical conditions on microbial communities and biogeochemical cycling, scientists have carried out extensive surveys of sediment microbial diversity. Unexpectedly diverse bacterial communities have been recorded in studies on sediments from ODP, IODP, and other expeditions (Marchesi et al., 2001; Inagaki et al., 2003, 2006; Kormas et al., 2003; Newberry et al., 2004; Webster et al., 2006, 2009; Parkes et al., 2005, 2014), and the global pattern of bacterial β-diversity in seafloor and seawater ecosystems was revealed with the development of next-generation sequencing methods (Zinger et al., 2011). Despite contrasting biodiversity in different oceanographic provinces, there are some clear common sub-seafloor bacteria, including the phyla *Chloroflexi*, *Gammaproteobacteria*, *Planctomycetes*, and candidate phylum JS1 (Webster et al., 2004; Fry et al., 2008). Principal components analysis and canonical analysis further indicated that prokaryotic composition may be linked to sediment type/habitat (Parkes et al., 2014).

However, environmental conditions are not the only factors that shape microbial communities across habitats (Zhang et al., 2014). In sediments, microbial distributions should also reflect microbial deposition from the upper water layers across both depth and time scales. Dispersal limitation in sediments would overall generate a negative correlation between compositional similarity and depth distance (a distance–decay relationship, Green and Bohannan, 2006; Hanson et al., 2012) or geological age distance between samples (a time–decay relationship). However, deep biosphere studies to date almost always focused on geochemical control of and interactions with microbial communities. Very few studies have addressed the relationship between microbial composition and depth distance or geological age distance. Such relation construction is crucial in determining to what extent the current microbial distributions could reflect deposition history and geological times.

The South China Sea (SCS), which is situated at the junction of the Eurasian, Pacific, and Indo-Australian plates, is one of the largest semi-enclosed marginal seas (Hu et al., 2000). Geographic location of the SCS deep basin in the tropical–subtropical western North Pacific make it a critical linkage to some of the major western Pacific tectonic units (Expedition 349 Scientists, 2013) and sensitive to climate system. Since the late Mesozoic, the SCS area has been experienced many first-order tectonic and paleoclimatic events (Expedition 349 Scientists, 2013). Extensive surveys of the microbial distribution and diversity in SCS water columns and surface sediments have been previously carried out (Wang et al., 2010; Ling et al., 2012; Li and Wang, 2013; Wang et al., 2014; Wu et al., 2014; Zhang et al., 2014, 2016). Seven bacterial 16S rRNA gene clone libraries from the Nansha coral-reef platform, the west slope, the north slope, and the Xisha trough have revealed that the SCS surface sediment bacterial community was dominated by *Planctomycetes*, *Deltaproteobacteria*, candidate division OP11, and *Alphaproteobacteria* (Li and Wang, 2013), which differs from other surface sediment bacterial communities in the continental margin along the Pacific Ocean (Fry et al., 2008). However, in cold seep sediments from the northern SCS, *Chloroflexi* and candidate phylum JS1 were predominant in the bacterial libraries (Zhang et al., 2012). To date, information regarding sedimentary microbial communities down to 800 m over approximately the past 20 million years has not yet been revealed.

Here, we investigated the distribution and diversity of the bacterial communities along a sediment core at site U1433 from International Ocean Discovery Program (IODP) expedition 349, which is at the southern center basin of the SCS, using sequences of the V3–V4 region of the 16S rRNA gene, as determined by Miseq. The objectives of this study were to (1) explore bacterial community variation across depth and times; (2) identify the possible geochemical factors that control the distribution of bacterial compositions and their interactions; and (3) evaluate the potential importance of environmental (geochemical) condition of habitat, geographic isolation across depth, and geological age in shaping microbial community structure in SCS sediments.

2. Materials and methods

2.1. Sample collection and environmental parameters

Sediments were collected from site U1433 in the SCS (hole U1433A, 12°55.1380′N, 115°02.8345′E; hole U1433B, 12°55.1313′N, 115°02.8484′E) in March 2014 during IODP Expedition 349. Site U1433 was cored into oceanic basement near the fossil spreading center, and spanned the Miocene to Pleistocene epochs. Hole U1433A was cored to refusal at 188.3 mbsf. Hole U1433B was drilled to 186.1 mbsf and then cored into basement, reaching a final depth of 858.5 mbsf. The sediment/basement interface occurred at approximately 798.5 mbsf. Fluid community tracers (FCT) (twenty samples) were used to measure contamination during drilling, and only samples with no detectable contamination were used for this study. Forty-nine subsamples were obtained and immediately stored at − 80 °C until further molecular analysis. All environmental variables used in this study, including depth and geological age, were downloaded from Li et al. (2015).

2.2. DNA extraction, PCR, and sequencing

DNA extractions were performed from 10 g (wet weight) of sediment by using the FastDNA® SPIN kit for Soil (MP Biomedicals, LLC, Solon, OH, USA) following the manufacturer's instructions and then stored at − 80 °C until use. The quality and quantity of DNA were assessed with a NanoDrop spectrophotometer (ND-2000, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the DNA concentrations were in the range of 3.6–32.8 ng/μL.

The bacterial hypervariable V3–V4 region of the 16S rRNA gene was amplified using specific primers with a unique barcode sequence at the 5′ end. PCRs were performed in 30-μL reaction mixtures with 15 μL Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Beverly, MA, USA), 0.2 μM forward primer 341F (5′-CCTAYGGGRBGCASCAG-3′), 0.2 μM reverse primer 806R (5′-GGACTACNNGGGTATCTAAT-3′), and approximately 10 ng template DNA. Thermal cycling consisted of an initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR products were mixed with an equal volume of 1 × loading buffer (which contained SYBR green) and separated by electrophoresis on a 2% agarose gel. Bands between 400 and 450 bp were excised and purified with a GeneJET Gel Extraction Kit (Thermo Scientific, San Jose, CA, USA).

Sequencing libraries were generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Beverly, MA, USA) based on the manufacturer's recommendations. Library quality was assessed by the Qubit 2.0 Fluorometer (Thermo Scientific, San Jose, CA, USA) and Agilent Bioanalyzer 2100 system. Finally, the libraries were sequenced on an Illumina MiSeq platform, and 300-bp paired-end reads were generated and assembled to form a longer sequence by the fast length adjustment of short reads (FLASH) software. Previously described criteria (Sogin et al., 2006) were used to assess sequence quality. Sequences that contained more than one ambiguous nucleotide (N) that did not have a complete barcode and primer at one end, or that were shorter than 350 bp after removal of the barcode and primer sequences were culled. The quality-filtered sequences were assigned to samples by examining the barcode.

2.3. Sequence processing

Quality-controlled sequences were analyzed in MOTHUR following standard operating procedures (http://www.mothur.org/wiki/MiSeq_SOP) (Schloss et al., 2011). Briefly, sequences were simplified into unique sequences with the *unique.seqs* command and then aligned to the SILVA database with the *align.seqs* command. The resulting alignment was then screened and filtered to ensure that all the sequences overlapped in the same alignment space. To further reduce sequencing errors and remove chimeras, the *pre.cluster*, *chimera.uchime*, and *remove.seqs* commands were used. Finally, classification was conducted using the MOTHUR version of the "Bayesian" classifier with the SILVA reference sequences, and the confidence cut-off was set to 60%. The sequences that were classified as "Cyanobacteria_Chloroplast," or "Mitochondria," or "unknown" (could not be classified at the kingdom level) were removed from the data set. Furthermore, sequences were clustered into operational taxonomic units (OTUs) at a 3% dissimilarity level, and all samples were analyzed at the same sequencing depth using the *sub.sample* command for normalization. Based on OTU assignment, diversity indices (inverse Simpson and Shannon) were calculated in MOTHUR. The GenBank submission number for the sequences from this study is SRP092136.

2.4. Statistical analysis

Non-metric multidimensional scaling (NMDS) was used to discriminate bacterial community composition between different samples based on Bray–Curtis similarities with PRIMER 5 (Clarke and Gorley, 2001). Similarities among different samples were presented in a multidimensional space by putting more similar samples closer together (Kruskal, 1964a, 1964b). One-way analysis of similarity (ANOSIM) in PRIMER 5 was used to test whether the spatial separation of different samples visualized in the NMDS plot were statistically significant (Clarke and Warwick, 1994). Similarity percentage (SIMPER) analysis in PRIMER was used to reveal which organisms were responsible for the similarity and dissimilarity observed in community composition between clusters.

Canonical correspondence analysis (CCA) was chosen as the multivariate method to analyze the correlations between bacterial community structure (based on the relative abundance of OTUs) and environmental variables (z-score transformation) with CANOCO (Ter-Braak, 1989), because the maximum gradient length of detrended correspondence analysis was longer than 3.0. Optimal CCA models were produced with "forward selection" via Monte Carlo permutation significance tests (999 permutations; default setting), and significant explanatory parameters ($P < 0.05$) without multicollinearity (variance inflation factor < 20) (Ter-Braak, 1986) were acquired.

Standard and partial Mantel tests were run in R (VEGAN package) to further assess correlations between environmental variation, depth distance, geological time distances between samples and bacterial community composition (based on the relative abundance of OTUs). The standard Mantel test estimates the correlation between two matrices, whereas the partial Mantel test estimates the correlation between two matrices, which controls for the effects of a third matrix. Dissimilarity matrices of communities were based on Bray–Curtis distances between samples. Environmental variables, depth, and age were normalized using z-score transformation, and Euclidean distances between different samples were calculated. Significance of the Mantel statistic based on Pearson's product-moment correlation was obtained after 999 permutations. The results of the statistical tests were assumed to be significant at $P \leq 0.05$.

2.5. Quantitative PCR targeting bacterial 16S rRNA gene

Quantitative PCR was carried out in 25-μL reaction mixtures using a 96-well Bio-rad CFX96 Real-Time PCR system (Bio-rad, Inc., Hercules, CA, USA). Reaction mixtures included 12.5 μL of SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China), 5 μg bovine serum albumin, 0.4 μM forward primer Bac349F (5′-AGG CAG CAG TDR GGA AT-3′), 0.4 μM reverse primer Bac806R (5′-GGA CTA CYV GGG TAT CTA AT-3′), and 1 μL template DNA. Thermal cycling consisted of denaturation at 96 °C for 25 s, and annealing and extension at every 1 °C intervals between 55 and 60 °C for 360 s for a total of 35 cycles. All samples were tested in triplicate. Standard curves were constructed using the plasmid DNA of *E. coli*. Using 10-fold increments, the standard concentrations were adjusted from 10^7 to 10^0 gene copies μL^{-1}. The amplification efficiencies were between 92.9 and 99.0%, with R^2 values > 0.99. Specificity of the quantitative PCRs was confirmed by melting curve analysis, agarose gel electrophoresis, and sequencing analysis.

3. Results and discussion

3.1. Contamination testing

The bacterial community DNA from FCT samples was compared to the same measurements of the core samples to check if the drilling fluids contain microbes that can be regularly tracked as recognizable contaminant taxa. Clustering analysis based on 2-D NMDS ordination and one-way ANOSIM showed that there were significant differences ($R = 0.359$, $P < 0.01$) in community structure between FCT and core samples (Suppl Fig. S2). However, the FCT sample at 166.52 mbsf clustered within the core samples. Hierarchical cluster dendrograms were further generated from the Bray–Curtis similarity matrix between whole-round and FCT samples (Suppl Fig. S3). The FCT sample at 166.52 mbsf was clearly distinct from the core sample at the same depth. The distinct community differences between whole-round and FCT samples revealed no apparent drilling fluid contamination of sampled core interiors.

3.2. Bacterial vertical distribution

Quantitative PCR data of the 16S rRNA gene showed that bacterial abundance rapidly decreased with depth, from 7.75×10^5 to 3.83×10^2 copies/cm^3. A sharp decrease of bacteria, nearly three orders of magnitudes decline, could be observed within the first 100 mbsf in the Pleistocene. The decline continued to the end of Pliocene, when the 16S rRNA gene abundance decreased to about 1.03×10^3 copies/cm^3. Then, the copy numbers became relatively stable in deeper parts during the Pliocene, late Miocene, and early to middle Miocene (Fig. 1A). Quantitative PCR results also showed that 16S rRNA gene abundance was significantly correlated with sampling depth (Log cells = 7.38–1.70 Log depth, $R^2 = 0.87$, $P < 0.0001$) according to logarithmic regression (Fig. 1B). The slope was relatively larger but most similar to that of South Pacific Gyre samples (Kallmeyer et al., 2012; Expedition 329 Scientists, 2011) among samples from the Atlantic Ocean, Mediterranean Sea, and Pacific Ocean sites (Parkes et al., 1994, 2014), although those slopes were based on regression of cell count and depth. When converting gene copies to counts, cell counts should be lower at site U1433 than those in the South Pacific Gyre, which have the lowest phytoplankton productivity and lowest sedimentation rates among oceans worldwide; thus, there are relatively lower cell abundances and greater attenuation with depth in sediments. This finding indicated that the southern center basin of the SCS might be fairly oligotrophic with low ocean primary production (Wang and Li, 2009).

3.3. Diversity analysis

A total of 1,047,695 bacterial reads were obtained from our survey, of which 975,741 were eligible for subsequent analyses. Sequences were grouped into OTUs with a 97% identity threshold. Diversity indices (inverse Simpson and Shannon) of all samples are shown in Fig. 2.

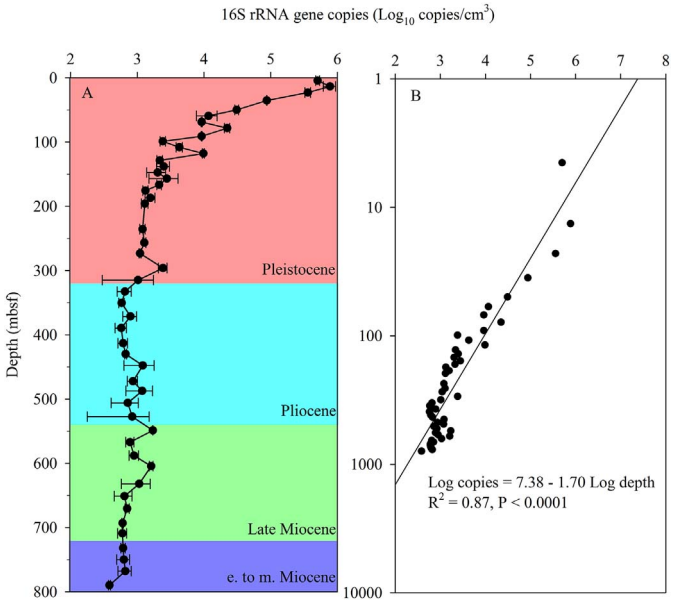

Fig. 1. Copy numbers of the bacterial 16S rRNA genes along the sediment core (A) and gene depth regression analysis (B) at IODP 349 site U1433 in the South China Sea.

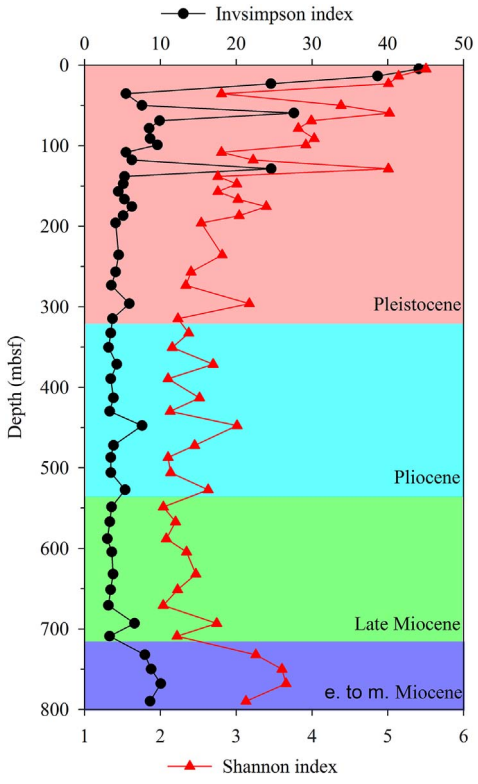

Fig. 2. Diversity indices (inverse Simpson and Shannon) for bacterial communities along the sediment core.

The highest diversity emerged at 4–129 mbsf in the Pleistocene, and lower diversity persisted from 130 mbsf (Pleistocene) to 732 mbsf (Miocene) at a relative stable inverse Simpson index range of 3.0–7.6, except for a slight rise (inverse Simpson indices of 8.0–10.0) between 732 and 790 mbsf in the early to middle Miocene (Fig. 2). Shannon indices, which ranged from 2.0 to 5.5, showed a similar pattern.

High bacterial diversity usually suggests an active community that responds to changing environmental conditions (Parkes et al., 2014). Because deposited organic matter and minerals may be activated from being recalcitrant at depth as temperatures increase due to a steep

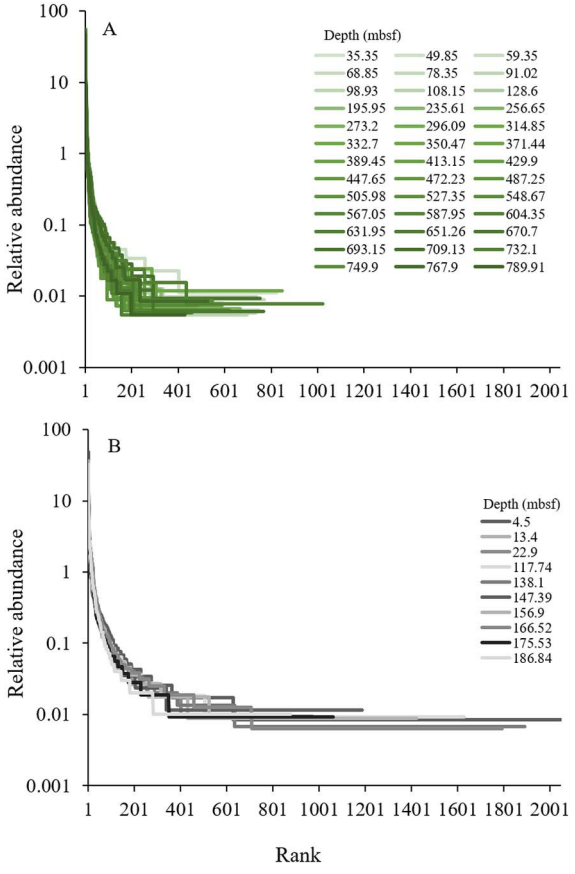

Fig. 3. Bacterial rank logarithmic OTU abundance for each sample at a 97% sequence identity cut-off. (A) Dominant OTUs are highly abundant at most depths; (B) the communities contain many OTUs with relatively low abundance in three of the nearest-surface sediment samples and seven samples between 100 and 200 mbsf in the Pleistocene.

geothermal gradient (Parkes et al., 2007), increasing diversity in the early to middle Miocene despite lower gene abundances might reflect enhanced activity in response to increasing temperature (approximately 65 °C at 800 mbsf; data not shown). Rarefaction curves of OTU number versus sampling effort were generated using a threshold value of 97%, and showed similar patterns with diversity indices. Additionally, they all showed an increasing slope, which indicates that sampling did not reach saturation in our tag sequences (Suppl Fig. S4).

In the bacterial 16S rRNA gene libraries, the top 10 abundant OTUs accounted for 67–91% of the total community at most depths (Fig. 3A), which indicates that dominant groups were highly abundant. However, in three of the nearest-surface sediment samples and seven samples between 100 and 200 mbsf in the Pleistocene, the 10 most abundant OTUs accounted for only 38–64% of total sequences and the communities stood out by containing many OTUs with relatively low abundance (Fig. 3B). In the deep biosphere, it has been proposed that rare groups include Bacteria and Archaea that are involved in sulphate reduction and methanogenesis (Fry et al., 2008); here, bacterial MiSeq sequencing data from site U1433 indicated that retrieved sequences present in low abundance were predominately related to species involving in sulphate reduction and nitrogen cycling, e.g. nitrification, denitrification, and anammox.

3.4. Variation in bacterial community composition across depths

Based on the relative abundance of OTUs, NMDS ordination analysis clearly revealed differentiation in the bacterial communities between different samples (Fig. 4). The cluster of core samples that spanned the Miocene to Pleistocene separated into two clusters of 4.5–98.93 mbsf (cluster I) and 108.15–789.91 mbsf (cluster II) at 81.13% dissimilarity

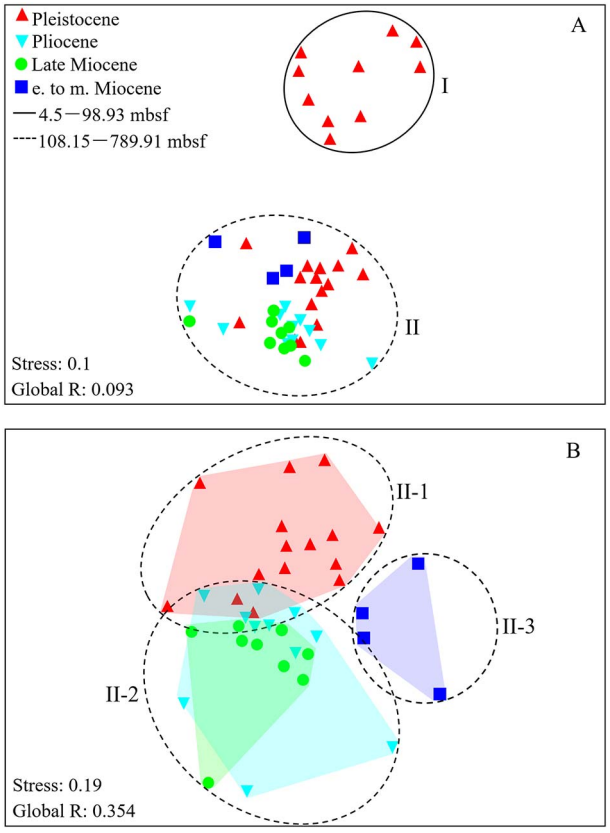

Fig. 4. Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis similarities among bacterial communities from all the four geological times. Pleistocene, red regular triangles; Pliocene, light blue inverted triangles; late Miocene, green circles; early to middle Miocene, dark blue squares. Each symbol represents an individual sample in the NMDS charts. Roman numerals represent cluster serial number. (A) All communities; (B) communities from cluster II in A.

(ANOSIM, $P < 0.05$; Fig. 4A). Moreover, cluster II further separated into three sub-clusters: the 108.15–314.85 mbsf cluster (II-1), the 332.7–709.13 mbsf cluster (II-2), and the 732.1–789.91 mbsf cluster (II-3) (Fig. 4B). Significant differences between pairwise clusters were obtained using ANOSIM ($P < 0.05$). NMDS analysis revealed that bacterial composition markedly changed over depth and geological time, which are linked to depth-specific geochemical and physical conditions, such as temperature, salinity, oxygen, nutrients, total organic carbon/nitrogen content, and mineralogy.

Analysis of 16S rRNA gene sequences from the sediment core (Fig. 5) revealed that *Proteobacteria* are particularly prevalent in SCS sediments. Dominant bacterial phyla included *Chloroflexi*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Deltaproteobacteria*, and *Betaproteobacteria*, with 3.5–66.4%, 4.3–24.6%, 2.2–27.2%, 2–27.3%, 2.3–11.5%, and 1.3–12.5% of 16S rRNA gene sequences, respectively. *Firmicute*, *Acidobacteria*, *Bacteroidetes*, *Nitrospirae*, candidate divisions OP9 and OP8, *Cyanobacteria*, candidate phylum BHI80–139, and *Gemmatimonadetes* were relatively less common, averaging 5%, 3%, 1.9%, 1.5%, 1.4%, 0.5%, 0.8%, 0.6%, and 0.5% of sequences, respectively. Although fairly low in abundance, *Deferribacteres* (averaging 0.4%), *Verrucomicrobia* (0.4%), *Planctomycetes* (0.2%), *Chlorobi* (0.2%), and *Epsilonproteobacteria* (0.09%) were consistently detected lineages. This is distinct from those seen in the SCS surface sediments, where *Planctomycetes* and candidate division OP11 were abundant (Li and Wang, 2013). In addition, a striking pattern was that the abundance of members affiliated with *Gammaproteobacteria*, *Actinobacteria*, and *Cyanobacteria* consistently decreased with increasing depth in general, whereas those affiliated with *Chloroflexi*, candidate division OP9, candidate phylum BHI80-139, and *Nitrospirae* increased (Fig. 5). Contradictory patterns were found in the Canterbury Basin and the Pacific Ocean off Japan, where *Chloroflexi* dominated microbial communities at shallow depths and decreased rapidly with depth, whereas proteobacterial relative abundance increased sharply with depth (Ciobanu et al., 2014; Inagaki et al., 2015). Parkes et al. (2014) found that subsurface sediment 16S rRNA gene libraries from organic-rich shelf/margin sites significantly correlated with high average percentages of *Chloroflexi*. However, the southern center basin of the SCS might be fairly oligotrophic, as indicated by gene depth regression, and thus had a distinctive pattern. Overall, the bacterial community composition corresponded well to different NMDS clusters, and the results clearly indicated the spatial dynamics of bacterial composition along with depth over geological time scales.

SIMPER analysis showed that the taxa responsible for the bacterial community differences (Suppl Tables S1, S2, S3 and S4) were dominated primarily by populations of *Gammaproteobacteria* (members of *Pseudomonadales*, *Enterobacteriales*, *Aeromonadales*, *Oceanospirillales*, and *Alteromonadales*), candidate division OP9, *Chloroflexi* (members of *vadinBA26*), *Cyanobacteria* (members of *Prochlorococcus*), *Nitrospirae* (members of *Nitrospira*), and *Actinobacteria* (members of *Actinobacteridae*). Cluster I (4.5–98.93 mbsf in the Pleistocene) was characterized by relatively high abundances of *Gammaproteobacteria* and *Cyanobacteria* (Suppl Tables S1; Fig. 5A and B). Cluster II-1 (108.15–314.85 mbsf in the Pleistocene) was characterized by relatively high abundances of *Gammaproteobacteria* and *Actinobacteria* (Suppl Tables S2 and S3; Fig. 5A), Cluster II-2 (332.7–709.13 mbsf in the Pliocene and late Miocene) was characterized by relatively high abundances of candidate division OP9, *Chloroflexi*, and *Nitrospirae* (Suppl Tables S2 and S4; Fig. 5A and B); and Cluster II-3 (732.1–789.91 mbsf in the early to middle Miocene) was characterized by relatively high abundances of candidate division OP9 and *Chloroflexi* (Suppl Tables S3 and S4; Fig. 5A and B). *Gammaproteobacteria*, *Chloroflexi*, and candidate division OP9 were major contributors to differences among clusters along the sediment core. This finding indicates that the differences in bacterial community composition between clusters were explained by the dominant groups present.

Gammaproteobacteria members usually exhibit broad ranges of aerobicity, trophism (including chemoautotrophism and photoautotrophism), and temperature adaptation (Scott et al., 2006), and drive important aspects of marine carbon and sulphur cycles in marine sediments (Dyksma et al., 2016). *Chloroflexi* was revealed to have potential roles in sediment carbon cycling by metagenomic analysis, including organohalide respiration, respiration of sugars, fermentation, CO_2 fixation, and acetogenesis with ATP formation by substrate-level phosphorylation (Hug et al., 2013). Candidate division OP9 was newly classified as candidate phylum "*Atribacteria*" (Carr et al., 2015), and is common in anoxic methane-rich sediments. A partial single cell genome indicated that members of the OP9 lineage exhibit fermentative and saccharolytic lifestyles (Dodsworth et al., 2013). The potential fermentation products may in turn support methanogens within the sediment microbial community (Carr et al., 2015), thus playing a potential role in carbon cycling in deep sediment. Cultured members of *Nitrospira* are known to microaerobically oxidize nitrite (Daims et al., 2016), and *Planctomycetes*, which were relatively low in abundance but widely distributed, have the capability of anaerobically oxidizing ammonia (Junier et al., 2010), which indicates that nitrification may be an important metabolic process in sedimentary prokaryotic communities of the SCS. In addition, sediment bacterial communities have potential roles linking to sulphur cycle, with consistent detection of *Deltaproteobacteria*, which contains major genera of sulphate reducers (Muyzer and Stams, 2008), and *Epsilonporteobacteria*, which includes several known lithotrophic sulphur-oxidizing species (Hubert et al., 2012).

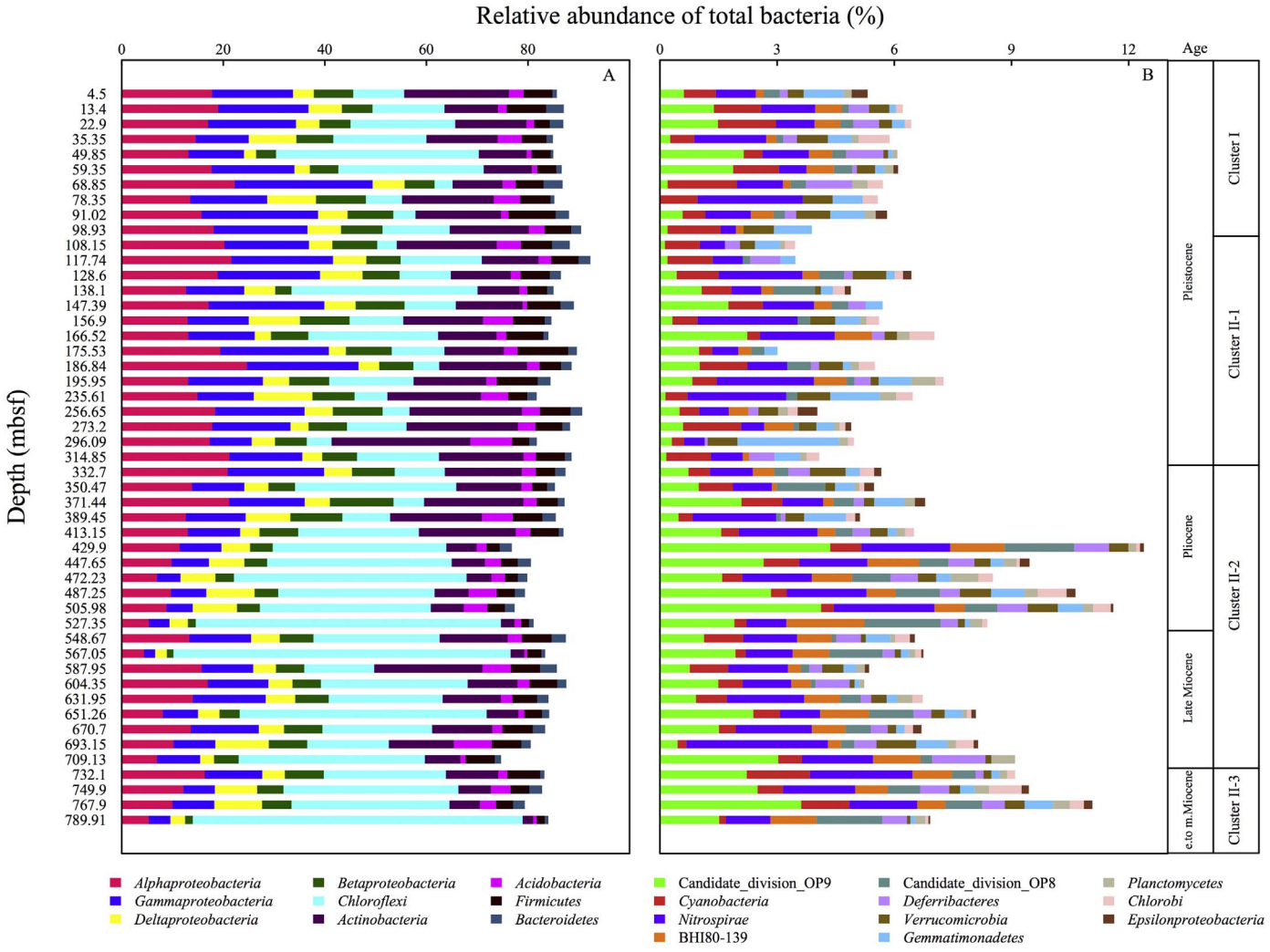

Fig. 5. Relative abundance of 16S rRNA gene sequences detected along the sediment core. (A) Communities with relative abundances > 1.9% (*Bacteroidetes*); (B) communities with relative abundances lower than 1.4% (Candidate division OP9).

3.5. Key factors that explain bacterial community composition variation across depths

CCA revealed that total organic carbon concentration (TOC, $P = 0.001$), ratio of total organic carbon:total nitrogen (C/N, $P = 0.001$), phosphate concentration ($P = 0.001$), and salinity ($P = 0.029$) were the statistically most significant variables, and explained approximately 36% of the total variance in the bacterial community composition over geological time scales (Fig. 6), which yielded a highly similar pattern to that of the NMDS analysis (Fig. 4). The first two CCA axes explained 89.2% of the cumulative variance of the 16S rRNA gene–environment relationship. Moreover, the bacterial communities from 35.35–138.1 mbsf in the Pleistocene (most belong to cluster I in Fig. 4A) were positively correlated with TOC, phosphate concentration, and salinity. Eight of 13 bacterial communities from 296.09–548.67 mbsf, spanning from the beginning of the Pleistocene to the end of the late Miocene, were positively correlated with C/N (Fig. 6). TOC was generally high at the shallower depths and decreased with depth, whereas C/N remained relatively constant until the end of the late Miocene, except for some abrupt high values between 296.09 and 548.67 mbsf (Li et al., 2015). This finding indicated that the quality of the organic matter changed from being relatively labile in the shallower sediments to more stable with increasing depth (Ciobanu et al., 2014), especially in high C/N samples that may contain highly refractory organic matter. Strong correlations between bacterial

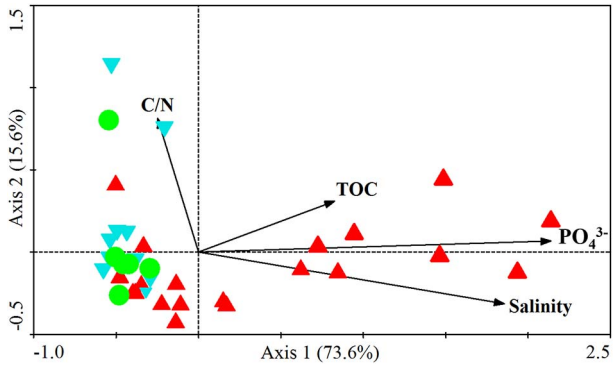

Fig. 6. Canonical correspondence analysis (CCA) of bacterial communities. Samples from 4.5, 13.4, 22.9, 117.74, 217.85, and 273.2 mbsf in the Pleistocene; 429.9 mbsf in the Pliocene, 670.7, 693.15, and 709.13 mbsf in the late Miocene; and 732.1, 749.9, 767.9, and 789.91 mbsf in the early to middle Miocene were excluded from CCA because of a lack of environmental variables. Pleistocene, red regular triangles; Pliocene, light blue inverted triangles; late Miocene, green circles. Each symbol represents an individual sample, and arrows represent statistically significant environment variables that explain the observed patterns ($P < 0.05$). TOC, total organic carbon concentration; PO_4^{3-}, phosphate concentration; C/N, ratio of total organic carbon: total nitrogen.

community composition, and TOC and C/N reinforce that prokaryotes can survive on buried organic matter in deep ancient deposits, and consequently, further change the type and quantity of the organic

Table 1
Mantel and partial Mantel test summary statistics. The *P*-values were calculated using the distribution of the Mantel or partial Mantel test statistics estimated from 999 permutations.

Samples	Mantel			Partial mantel[a]					
	R (env)	R (depth)	R (age)	R (env, depth)	R (env, age)	R (depth, age)	R (depth, env)	R (age, depth)	R (age, env)
All	0.4194**	0.3728**	0.1571	0.2486*	0.4186**	0.4862**	0.1309*	− 0.3674	− 0.1444
Pleistocene	0.4871**	0.5947**	0.4155**	0.09808	0.3609**	0.7671**	0.2908**	− 0.6878	− 0.02334
Plio.–Mio.	− 0.1533	0.1621	0.2609	− 0.07882	− 0.06387	− 0.05768	− 0.08518	0.2147	− 0.1083

* $P < 0.05$.
** $P < 0.01$.
[a] The "third" matrix in partial mantel tests are after comma in brackets.

matter over geological time scales. Organic carbon content has also been considered a control factor for biogeographical and vertical distribution of bacterial communities in previous studies, such as in SCS surface sediments, at a hydrate-containing site of the Andaman Sea, and in the North Sea (Sapp et al., 2010; Briggs et al., 2012; Li and Wang, 2013).

Mantel and partial Mantel tests were run in R (VEGAN package) to further estimate correlations between environmental variation, depth distance, or geological time scale and bacterial community composition (based on the relative abundance of OTUs) (Table 1). For all samples over the four geological times, Mantel ($P < 0.01$) and partial Mantel tests ($P < 0.05$) both indicated that bacterial community differences were significantly correlated with environmental variation and depth distance between sites. When samples were divided into Pleistocene and Pliocene to Miocene geological times, the changes in community compositions in the Pleistocene were only strongly correlated with depth distance ($P < 0.01$), whereas environmental variation was not significant based on a partial Mantel test that constrained the effects of depth (Table 1). There was no significant correlation in the Pliocene to Miocene geological times. It is notable that geological time scales did not correlate with bacterial community variation, as revealed by partial Mantel tests. These results indicated that, although environmental variables partially shaped the bacterial community composition, geographic isolation is relatively more important than environmental condition and geological age in developing unique marine sediment community structure, particularly in the Pleistocene.

4. Conclusions

We report the first hand data of the distribution and diversity of the SCS sedimentary microbial communities down to 800 m over approximately the past 20 million years to reveal how geological processes, deposition history, and environmental factors might have shaped the current microbial community in this subsurface biosphere. The bacterial 16S rRNA gene copies declined sharply with depth, and the regression of the cell (gene) and depth is relatively most similar to that of South Pacific Gyre samples among samples from oceans worldwide, which indicates that the sampling location may be fairly oligotrophic with low ocean primary production. There was an unexpected increase of community diversity in the early to middle Miocene, which probably reflects enhanced activity in response to increasing sediment temperature. Spatial variations based on cluster analysis in bacterial communities were related to depth and geological time. The retrieved sequences affiliated with *Gammaproteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Chloroflexi*, candidate division OP9, candidate phylum BHI80-139, and *Nitrospirae* significantly contributed to differences among clusters. As indicated by sequence classification, microbial community potentially played roles in biogeochemical processes of SCS sediment, such as organic matter degradation, nitrification, sulphur oxidization, and sulphate reduction. Strong correlations between bacterial community composition, and TOC and C/N highlighted that microorganisms can survive on buried organic matter in deep ancient deposits and finally change the type and quantity of the organic matter over geological time scales. Although environmental variables partially shaped the bacterial community composition, partial Mantel tests between matrices revealed that geographic isolation across depths is relatively more important for developing unique community structure in marine sediments than environmental condition and geological age. Microbial deposition from the upper water layer introduced ancient species into the sediment, and distinct geographic isolation resulting from strong dispersal limitation makes communities develop on unique ecological trajectories to produce unique communities.

These findings provide a comprehensive understanding of factors that control the microbial community composition in the SCS sediment core, and highlight the importance of determining the relationship between microbial composition and depth distance or geological age distance in addition to geochemical conditions. Clarifying such relationships is crucial for determining to what extent the current subseafloor biosphere could reflect deposition history and geological history.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.margeo.2017.06.010.

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