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RESEARCH ARTICLE

Abundance and diversity of candidate division JS1- and *Chloroflexi*-related bacteria in cold seep sediments of the northern South China Sea

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Abstract Candidate division JS1-and *Chloroflexi*-related bacteria are ubiquitous in various deep marine sediments worldwide, yet almost nothing is known about their abundance and diversity in cold seep sediments. Here, we investigated the abundance and diversity of JS1- and *Chloroflexi*-related bacteria in a cold seep marine sediment core collected from the northern South China Sea (SCS) with the employment of quantitative polymerase chain reaction (qPCR) and 16S rRNA gene phylogenetic analyses. The qPCR results showed that 16S rRNA gene copies per gram of sediments for the total bacteria and JS1- and *Chloroflexi*-related bacteria were at magnitudes of 10^8 and 10^6, respectively. The relative abundance of JS1- and *Chloroflexi*-related 16S rRNA genes to that of total bacteria was 0.07%–8.78% throughout the core. Phylogenetic analyses showed that the JS-1 related clone sequences were dominant throughout the core. Our study provided insights into abundance and diversity of JS1- and *Chloroflexi*-related bacteria in the northern SCS cold seep sediments.

Keywords candidate division JS1, *Chloroflexi*, cold seep sediments, South China Sea

1 Introduction

Marine sediments are one of the major ecosystems on Earth and microbes are ubiquitous in such environments (Whitman et al., 1998; Parkes et al., 2000). Microbial composition and activity in the deep marine subsurface is one important focus in the field of environmental microbiology. Over the past decades, 16S rRNA gene-based molecular studies have revealed the frequent retrieval of many phylogenetic microbial groups in different marine sediments (Webster et al., 2004). For example, candidate division JS1 (Rochelle et al., 1994) and *Chloroflexi* (Yamada and Sekiguchi, 2009) are two of such phylogenetic groups.

The first sequence of candidate division JS1 was retrieved from subseafloor sediments at 78 m below the seafloor in the Japan Sea (Rochelle et al., 1994). Subsequently, large amounts of JS1 sequences were retrieved from various marine environments, such as mud volcanoes, cold seep sediments, methane hydrate-bearing sediments, hydrothermal vents, and benzene-degrading and acetate-utilizing sulfate-reducing sediments (Phelps et al., 1998; Inagaki et al., 2002; Teske et al., 2002; Inagaki et al., 2006; Niemann et al., 2006; Webster et al., 2006b). Therefore the JS1 members were thought to constitute a ubiquitous bacterial lineage within the deep subseafloor biosphere (Teske, 2006). However, none of the JS1 representatives has been obtained in culture and isolated successfully. Therefore their metabolic and physiologic functions remain unknown although one study currently showed that the JS1 bacteria can be heterotrophically enriched from sediment environments using acetate and glucose as substrates (Webster et al., 2011).

The phylum *Chloroflexi*, originally named as 'green

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nonsulfur bacteria', has been recognized as a typical ubiquitous bacterial taxon containing a number of diverse environmental 16S rRNA gene clones and playing significant roles in the environment (Rappé and Giovannoni, 2003). This phylum can be divided into at least six major classes: *Chloroflexi*, *Thermomicrobia*, *Anaerolineae*, *Caldilineae*, '*Dehalococcoidetes*', and unclassified subphylum IV (Blazejak and Schippers, 2010). The 16S rRNA gene sequences of the *Chloroflexi* frequently obtained from subsurface sediments fall into the classes *Anaerolineae*, *Caldilineae*, '*Dehalococcoidetes*', and the unclassified subphylum IV (Coolen et al., 2002; Reed et al., 2002; Inagaki et al., 2003; Newberry et al., 2004; Inagaki et al., 2006; Webster et al., 2006a). Cultivated members of the classes *Anaerolineae* and *Caldilineae* have been shown to degrade carbohydrates and amino acids under anaerobic conditions (Yamada and Sekiguchi, 2009), and the latest studies demonstrate these two classes of bacteria can use acetate as an energy source (Webster et al., 2011). Due to the limited number of cultured representatives of these classes in marine sediments, little is known about the physiologic properties of the *Chloroflexi* members.

The 16S rRNA gene clone sequences of the JS1- and *Chloroflexi*-related bacteria were frequently retrieved from various marine environments using universal 16S rRNA gene primers for polymerase chain reactions (PCR) (Teske et al., 2002; Kormas et al., 2003; Newberry et al., 2004; Alain et al., 2006; Inagaki et al., 2006; Webster et al., 2006a). One previous study showed that the JS1- and *Chloroflexi*-related 16S rRNA gene sequences were dominant in gas hydrate-bearing sediments (Inagaki et al., 2006), suggesting that these bacteria might play an important role in biogeochemical processes related with gas hydrates. Recently, Blazejak and Schippers (2010) designed a new specific PCR primer set and employed the quantitative PCR (qPCR) technique to quantify the JS1- and *Chloroflexi*-related bacteria, and their quantitative results indicated that these bacteria might dominate the bacterial communities and play an important role in subsurface and deeply buried marine sediments.

Cold seeps represent one of the most extreme marine environments. It has been a long-standing challenge in the field of microbial ecology and evolution to determine the microbial diversity and microbial community structures in cold seep sediments (Li et al., 1999). Although the JS1- and *Chloroflexi*-related bacteria are found to be widespread in marine sediments, almost little is known about their abundance and diversity in cold seep environments. Here, we analyzed the abundance and diversity of these two groups of bacteria in cold seep marine sediments collected from the northern South China Sea (SCS) by using techniques of qPCR and 16S rRNA gene phylogenetic analysis.

2 Materials and methods

2.1 Site description and sample collection

Since the first evidence of cold seeps was discovered by the Chinese research vessel "Haiyang 4" at the northern SCS, the area was named as "Haiyang 4 Area" by the "SONNE" 177 (SO177) cruise in 2004. Over the past decade, numbers of geophysical, geological, biologic and geochemical investigations have been performed in the northern SCS, and they provided evidences showing the presence of gas hydrate. The reported evidences included bottom simulating reflectors (BSRs) (Song et al., 2001), methane-derived cold seep carbonates and related microbial communities (Chen et al., 2005; Su et al., 2008), cold seep bivalves communities, bacterial mats, and alive bivalve samples obtained by "Haiyang 4" in 2004, and abundant dead bivalve shells collected by "SO177" in 2005 (Suess, 2005; Huang et al., 2008).

In summer 2006, a sediment core was collected at the "Haiyang 4 Area" of the northern SCS using a gravity piston corer (Fig. 1). The sampling site has a water depth of 3016 m. The core was immediately dissected into 50 cm sections and named according to the depth (from DSH1 to DSH12) as soon as it was onboard. The top 5 cm of each section was collected for this study. The collected samples were stored in dry ice on board and then were stored at −80°C in the laboratory until further analysis.

2.2 Geochemical analyses

Headspace methane concentration in the sediment core was measured using HP5890 Series II Gas Chromatography onboard (Su et al., 2007). Other geochemical parameters including alkalinity, chloride, ammonia, phosphate, hydrogen sulfide, and sulfate of the sediment were measured using previously published methods (Table 1) (Suess, 2005). Total organic carbon (TOC) in the sediment was measured according to procedures described elsewhere (Liu, 1996).

2.3 DNA extraction, PCR amplification, and phylogenentic analysis

DNA was isolated from 0.5 to 1 g samples (wet weight) of the sediment core at six depths using a FastDNAs Spin for Soil Kit (MP Biomedicals, Solon, USA) according to the manufacturer's instructions. Total DNA was quantified by using the NanoDrop 1000 spectrometer (Thermo Scientific, Wilmington, USA) and amplified using 16S rRNA gene primers 63F and 665R (Webster et al., 2004). PCR amplification conditions were as follows: 95°C denaturing 7 min, and then 35 cycles of 94°C denaturing 30 s, 62°C annealing 30 s, and 72°C extending 60 s, followed by a final extension step of 72°C for 10 min.

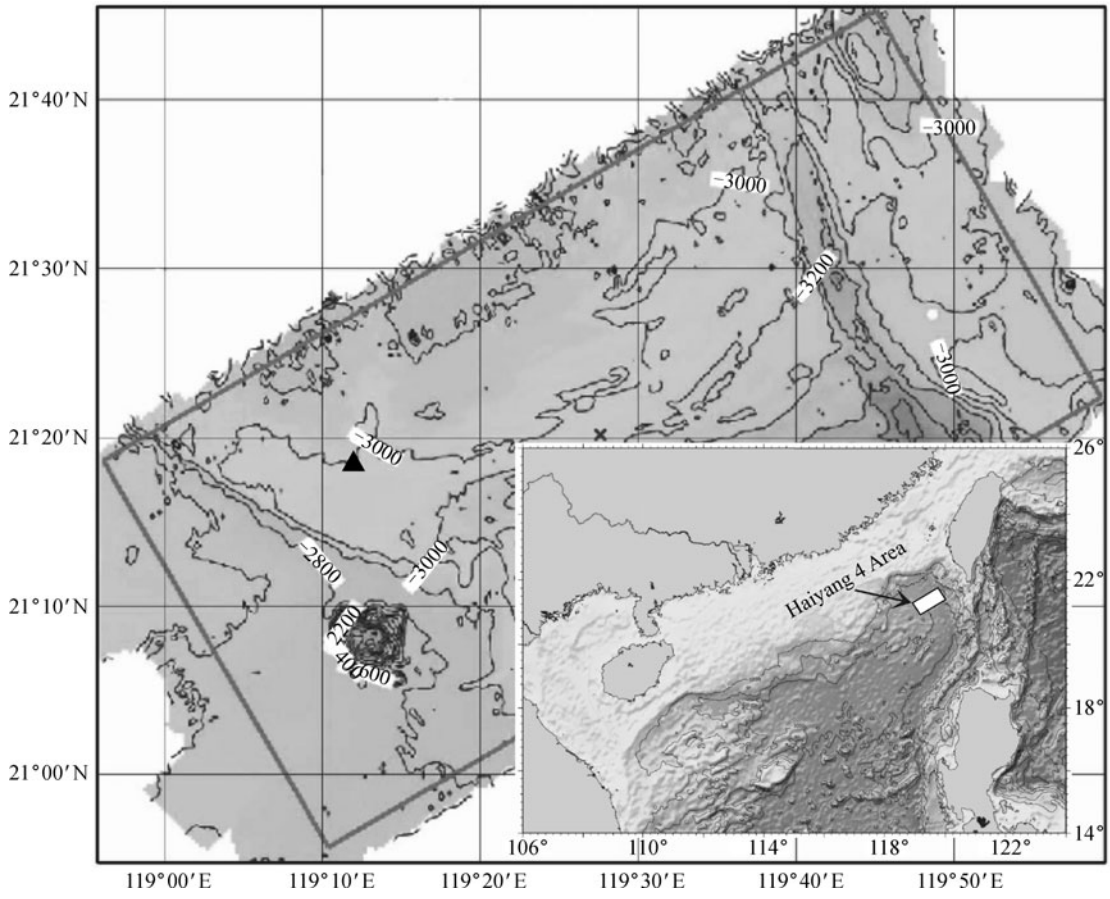

Fig. 1 Sampling site (▲) of the cold seep gravity core from "Haiyang 4 Area" of the northern South China Sea

Table 1 Methods used for geochemical analyses

Constituent	Method	References
Ammonium	Titration	Grasshoff et al. (1999)
TOC	Titration	Liu (1996)
Hydrogen sulphide	Spectrophotometry	Grasshoff et al. (1999)
Phosphate	Spectrophotometry	Grasshoff et al. (1999)
Headspace methane	Gas chromatography	Niewöhner et al. (1998)
Sulfate, chloride	Ion chromatography	http://wwwodp.tamu.edu/publications/notes/tn15/f_chem3.html

Note: Methods for pore water analyses were cited from the SO177 report (Suess, 2005)

PCR products obtained from each depth were purified and subsequently ligated into the pGEM-T Easy vector system (Promega, USA), and then transformed into JM-109 competent cells (Takara, Japan) according to the manufacturer's instruction. Clones were randomly picked and amplified using M13 primers. The selected clones were then sequenced with primer 63F. Nucleotide sequences were assembled and edited by using Sequencer v.4.8 (GeneCodes, Ann Arbor, MI). Operational taxonomic units (OTUs) were determined using DOTUR (Schloss and Handelsman, 2005) with a 97% cutoff value. The neighbor-joining tree was constructed using MEGA (molecular evolutionary genetics analysis) program version 4, and 1000 bootstrap replications were assessed. The sequences are available from the GenBank under the following accession numbers: JF506041–JF506066.

2.4 Quantification of JS1- and *Chloroflexi*-related bacteria and total bacteria

qPCRs were performed to assess the abundance of 16S rRNA genes for JS1- and *Chloroflexi*-related bacteria and

total bacteria. An ABI 7500 real-time PCR system was employed in the qPCR analysis (Applied Biosystems). The 16S rRNA gene abundance of total bacteria and JS1- and *Chloroflexi*-related bacteria were determined in 12 sediment samples (from DSH1 to DSH12, including the six samples for clone library construction, see Table 2). PCR products of the 16S rRNA genes from the same environmental samples using primers 27F/1492R were used as standards, which with serial dilutions (1∶10) were used to yield standard curve for total bacteria. Products of JS1 clone sequence DSH12B2 (GU475357) were used as JS1- and *Chloroflexi*-related bacteria standards. 16S rRNA gene primers specific for bacteria (331F/797R) (Nadkarni et al., 2002) and for JS1- and *Chloroflexi*-related bacteria (519F/665R) (Blazejak and Schippers, 2010) were employed for quantification of total bacteria and JS1- and *Chloroflexi*-related bacteria. The qPCR assay was performed using Power SYBR Green PCR Master Mix (UK). Amplification conditions were 50°C for 2 min, and then 95°C for 10 min, followed by 40 cycles of 30 s at 94°C, 30 s for annealing at 60°C (total bacteria) and 62°C (JS1- and *Chloroflexi*-related bacteria), 60 s for extension at 72°C, in a reaction volume of 20 μL, containing 10 μL of SYBR Green Mix (UK), 10 pmol of each primer, and 1μL template.

2.5 Statistical analysis

Canonical correspondence analysis (CCA) was performed using Canoco for Windows version 4.5 to assess the correlation between environmental factors and communities (OTU-level) of JS1- and *Chloroflexi*-related bacteria. The Mantel test was performed to assess the correlation between biotic and environmental matrices by using the *zt* software[1)] according to procedures as previously described (Jiang et al., 2009).

3 Results

3.1 Geochemical results

Headspace methane concentration in the collected sediment core was about 2.1 μmol/L at the top, and increased with depth, reaching to > 20.4 μmol/L at the bottom of the core. Sulfate concentration was 29.1 mmol/L at the top. It was stable at 28.2–28.7 mmol/L between the depths of 50–255 cm, and then decreased from 28.5 (at the 255 cm depth) to 12.6 mmol/L at the bottom (at the 555 cm depth) of the core. Correspondingly, hydrogen sulphide concentration varied with depth: the H_2S concentration was zero from the top to the 255 cm depth, and then abruptly increased to 1612 μmol/L at the bottom (555 cm depth) of the core. In general, the concentrations of ammonium and phosphate increased from the top to the bottom of the core. The concentration of Cl^- was about 550 mmol/L throughout the collected core, a little lower than the average concentration value of seawater. The TOC content was 0.5%–0.8% (dry weight) throughout the core (Table 3).

Table 2 qPCR-based 16S rRNA gene abundances of total bacteria and JS1- and *Chloroflexi*-related bacteria in cold seep sediments of the northern SCS

Sample number	16S rRNA gene copies per gram of sediments		
	Total bacteria (10^7)	Total JC[a)] (10^5)	Total JC/Total bacteria[b)]/%
DSH1	186.71 (± 16.94)	19.10 (± 1.48)	0.10 (± 0.01)
DSH2	17.41 (± 9.70)	36.06 (± 18.95)	3.00 (± 2.86)
DSH3	728.80 (± 25.40)	6.08 (± 1.25)	0.08 (± 0.00)
DSH4	69.09 (± 11.75)	5.26 (± 2.09)	0.07 (± 0.03)
DSH5	3.82 (± 2.62)	4.71 (± 2.03)	2.32 (± 2.31)
DSH6	4.34 (± 3.41)	3.68 (± 2.53)	2.21 (± 3.00)
DSH7	2.49 (± 1.29)	0.75 (± 0.11)	0.41 (± 0.25)
DSH8	0.84 (± 0.20)	7.25 (± 1.41)	8.78 (± 3.11)
DSH9	2.16 (± 0.11)	4.02 (± 4.02)	1.87 (± 2.16)
DSH10	1.20 (± 0.10)	1.72 (± 0.07)	1.57 (± 0.19)
DSH11	2.53 (± 0.93)	15.60 (± 8.88)	7.14 (± 5.84)
DSH12	3.17 (± 1.46)	1.24 (± 0.33)	0.49 (± 0.31)

Notes: a) candidate division JS1- and *Chloroflexi*-related bacteria; b) average of triplicates

1) http://www.psb.ugent.be/~erbon/mantel/

Table 3 Geochemical parameters of the sediment core

Sample number	Depth/(cmbsf)	SO_4^{2-}/(mM)	CH_4/(μM)	H_2S/(μM)	NH_4^+/(μM)	HPO_4^-/(μM)	Cl^-/(mM)	TOC/%
DSH1	5–10	29.1	2.05	0	6.60	6.00	555	0.51
DSH2	50–55	28.2	2.32	0	39.71	6.69	551	0.51
DSH3	100–105	28.3	2.53	0	40.67	6.69	558	0.56
DSH4	150–155	28.7	2.61	0	28.54	3.59	556	0.65
DSH5	200–205	28.6	1.59	0	45.82	4.91	557	0.58
DSH6	250–255	28.6	1.77	0	48.26	5.53	556	0.79
DSH7	300–305	28.5	2.49	14.80	56.29	3.98	554	0.61
DSH8	350–355	28.4	3.74	17.20	66.41	4.05	554	0.61
DSH9	400–405	25.8	5.45	5.60	92.15	6.31	556	0.50
DSH10	450–455	21.4	8.08	1276.80	100.40	10.12	554	0.55
DSH11	500–505	15.3	12.73	1824.00	179.80	16.19	546	0.59
DSH12	550–555	12.6	20.34	1612.00	203.10	17.67	549	0.56

3.2 Abundance of 16S rRNA gene of total bacteria and JS1- and *Chloroflexi*-related bacteria

The qPCR data showed that the abundances of total bacteria and JS1- and *Chloroflexi*-related bacteria decreased with depth, and these two bacterial groups were present in a significantly low abundance and only a minor part of total bacteria community. For example, above 55 cmbsf (centimeters below seafloor) depth, the 16S rRNA gene abundance of JS1- and *Choroflexi*-related bacteria were 1.91×10^6 to 3.61×10^6 copies g^{-1} sediment, which accounted for 0.1%–3% of the total bacterial 16S rRNA genes. In contrast, below this depth the abundance decreased to 0.75×10^5 to 1.56×10^6 copies g^{-1} sediment, which accounted for 0.07%–8.78% of the total bacterial 16S rRNA genes (Table 2 and Fig. 2).

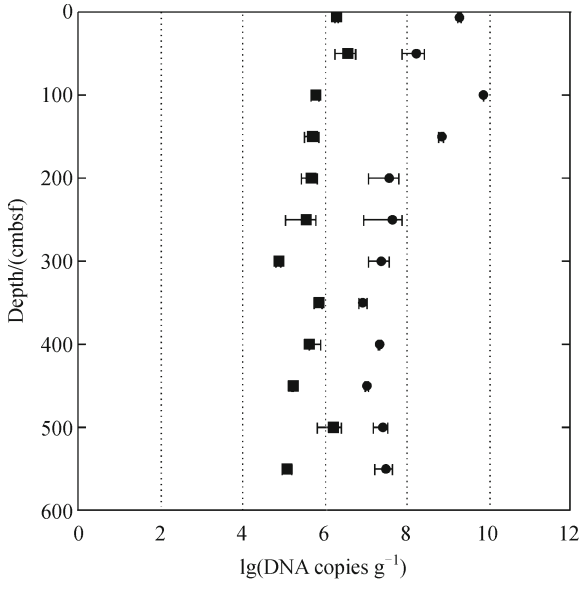

Fig. 2 DNA copy numbers of the 16S rRNA genes of the total bacteria (●) and JS1- and *Chloroflexi*-related bacteria (■) in the cold seep sediment core from the northern SCS

3.3 Diversity of JS1- and *Chloroflexi*-related bacteria

Six clone libraries were constructed targeting JS1- and *Chloroflexi*-related bacteria. 295 clone sequences were obtained and subjected to phylogenetic analyses (Table 4). 233 clone sequences were classified into JS1- and *Chloroflexi*-related bacteria (Fig. 3). Other sequences belonged to *Gammaproteobacteria* and *Deltaproteobacteria*. Among the JS1- and *Chloroflexi*-related clone sequences, 218 clone sequences were classified into candidate division JS1, and 15 clone sequences were affiliated with *Anaerolineae*, one of the named classes within the *Chloroflexi* phylum (Fig. 3).

The majority of JS1-related clone sequences (except one clone sequence, JC-DSH12-55, from the DSH12 library) obtained in this study were classified into one cluster, closely related to clone sequences retrieved from different marine environments, such as SCS subseafloor (clone MD2900-B17, EU386091) and Juan de Fuca segments Mothra sediments (clone Mothra_B6-29, GQ267094), Japan Trench (Li et al., 1999) and Gulf of Mexico cold seep sediments (Orcutt et al., 2010), and Nankai Trough (Reed et al., 2002) and Cascadia Margin methane hydrate-bearing sediments (FJ873257). The clone sequence JC-DSH12-55 was closely related (identity = 99%) to one clone sequence (ODP1230B32.21, AB177206) retrieved from methane hydrate-containing sediments in Peru Margin (Inagaki et al., 2006).

Seven, six, and two clone sequences belonged to the class *Anaerolineae* were detected at 5–10 cmbsf, 100–105 cmbsf, and 300–305 cmbsf, respectively (Fig. 3). They were related to the sequences derived from the seafloor

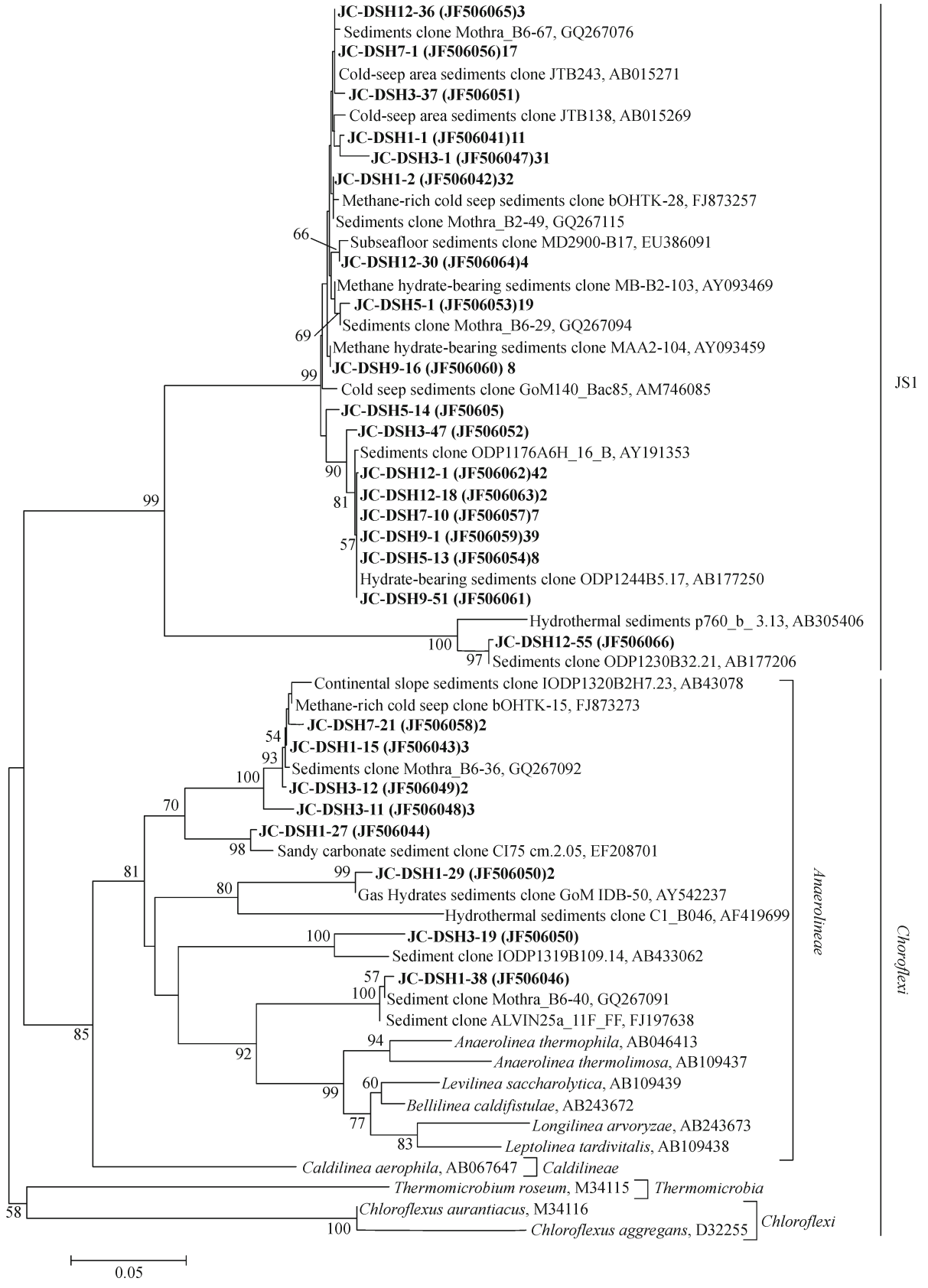

Fig. 3 Neighbor-Joining tree showing the phylogenetic relationships of candidate division JS1 and *Chloroflexi*-related bacteria 16S rRNA gene sequences cloned a cold seep sediment core from of the northern SCS. Sequences obtained in this study are marked in bold. One representative clone type within each phylotype is shown, and the number of clones within each phylotype is shown after the GenBank accession number. Bootstrap values of > 50% (for 1000 iterations) are shown.

Table 4 Clone library analyses of JS1- and *Chloroflexi*-related bacteria in cold seep sediments from northern SCS

Clone library	Number of clone sequences			
	No. of total clone sequences obtained	JS1-related bacteria	*Anaerolineae*-related bacteria	Other bacteria
DSH1	54	43 (80%)	7 (13%)	4 (7%)
DSH3	47	23 (49%)	6 (13%)	18 (38%)
DSH5	44	28 (64%)	0	16 (36%)
DSH7	47	24 (51%)	2 (4%)	21 (45%)
DSH9	50	48 (98%)	0	2 (4%)
DSH12	53	52 (98%)	0	1 (2%)

[e.g., clone ALVIN25a_11F_FF (FJ197638) and clone IODP1319B109.14 (AB433062)], methane-rich cold seep (clone Bhotk-15, FJ873273), and gas hydrate-containing sediments (clone GoM IDB-50, AY542237). No *Chloroflexi*-related clone sequences were retrieved below the 305 cm depth of the sediment core.

3.4 Statistical analysis

The simple Mantel tests result showed that total abundance of bacteria and the bacterial groups JS1 and *Anaerolineae* were not correlated with any environmental factors (Table 5). The TOC content was the only environmental factor that was negatively correlated ($r = -0.454$, $P = 0.047$) with the JS1- and *Anaerolineae*-related bacterial communities (OTU-level) (Table 5).

4 Disscussion

4.1 JS1- and *Chloroflexi*-related bacteria in cold seep sediments of the northern SCS

The relative abundance of JS1- and *Chloroflexi*-related bacteria in the cold seep sediments in the northern SCS was at the same magnitude as that in the methane-rich sediments, such as Peru Margin. In this study, the ratio of 16S rRNA genes of JS1- and *Chloroflexi*-related bacteria to total bacteria was up to 8.78%, within the range ($<$ 5%–10%) for the relative abundance of JS1- and *Chloroflexi*-related bacteria in Peru Margin (Blazejak and Schippers, 2010).

In addition, previous studies showed that 16S rRNA gene sequences of JS1-related bacteria were frequently recovered from cold seeps [e.g., Japan Trench (Inagaki et al., 2002), Gulf of Mexico (Orcutt et al., 2010)], and/or methane hydrate-bearing sediments [e.g., Peru Margin (Inagaki et al., 2006), Nankai Trough (Reed et al., 2002), indicating that JS1-related bacteria may prefer methane-rich environments. It has been confirmed that the sediments in the cold seep area of the northern SCS contained high concentration of methane (Suess, 2005; Huang et al., 2008). In Peru and Cascadia Margin hydrate-bearing sediments, JS1 group was the key bacterial representatives with$>$ 50% clone sequences (Inagaki et al., 2006). The ubiquity of JS1-related bacterial 16S rRNA gene sequences in sediments with high-concentration methane suggested this group bacteria may play an important role in methane cycling in sub-seafloor environments. However, so far the JS1 representatives have never been isolated in culture, and thus their metabolic pathway is still unknown. So further investigation is required to

Table 5 Simple Mantel tests for similarity between biotic and environmental matrices in cold seep sediments from the northern SCS

Environmental variables	Total bacteria[a)]		Total JC[b)]		OTU level		Major group level	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
CH_4	−0.105	0.334	0.156	0.210	0.464	0.206	0.056	0.400
Cl^-	−0.136	0.199	0.004	0.433	0.370	0.247	0.104	0.400
HPO_4^-	−0.158	0.132	0.084	0.265	0.454	0.288	0.034	0.400
H_2S	−0.082	0.329	0.125	0.233	0.504	0.158	−0.002	0.600
NH_4^+	0.114	0.184	0.162	0.159	0.582	0.056	0.101	0.200
SO_4^{2-}	−0.090	0.321	0.122	0.212	0.482	0.160	0.058	0.400
TOC	−0.112	0.321	−0.151	0.251	**−0.454**	**0.047**	−0.258	0.400

Notes: a) abundance of total bacteria; b) abundance of candidate division JS1- and *Chloroflexi*-related bacteria. *r*: the correlation value; + and − : positive and negative relationship, respectively; *P*: the correlation is significant when the *P* value is less than 0.05 (bold)

figure out the role of JS1-related bacteria in methane cycling of sediments.

4.2 Environmental factors affecting JS1- and *Chloroflexi*-related bacteria

Among the characterized environmental factors, no distinct correlation was found between the JS1- and *Anaerolineae*-related bacterial community and any single characterized environmental parameters (data not shown). However, the results of simple Mantel test showed that only TOC negatively affected the JS1- and *Anaerolineae*-bacterial groups (at the OTU level), indicating that there may be high diversity of the JS1- and *Anaerolineae*-related bacteria when TOC content is low and vice versa. This finding was inconsistent with previous studies in that JS1-related bacteria were considered to prefer sedimentary habitats with organic-rich, low sulfate (Webster et al. 2007) and/or high concentrations of methane (Inagaki et al., 2006). The reasons for the observed inconsistency await further investigation.

In summary, the JS1- and *Chloroflexi*-related bacteria were abundant (10^5 to 10^6 16S rRNA gene copies per gram of sediment) in the northern SCS cold seep sediments, consistent with that found in other methane-rich sediment. These two groups of bacteria may play an important role in methane cycling in the cold seep sediments of the northern SCS.

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