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Bacterial diversity in Arctic marine sediment determined by culture-dependent and -independent approaches

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Abstract Bacterial diversity in surface sediment from the Arctic Ocean was investigated by culture-dependent and -independent approaches. Conventional culture-dependent techniques revealed 11 strains based on their distinct morphological characteristics on marine Zobell 2216E agar plates. Phylogenetic analysis showed that these isolates belonged to three major lineages of the Bacteria, γ-proteobacteria, Bacteroidetes and Actinobacteria, and that they included 10 genera. Most isolates were psychrotrophic, and NaCl was not necessary for their growth. Furthermore, they exhibited activity of at least one extracellular hydrolytic enzyme at 4°C and had various abilities to assimilate carbon sources. A total of 67 phylotypes were detected among 142 clones based on the 16S rRNA library of the total community DNA and grouped into nine major lineages of bacteria. Phylotypes affiliated with γ-, δ- and ε-proteobacteria accounted for 36.7%, 21.8% and 16.9% of the total clones, respectively. The rest of the clones belonged to Bacteroidetes, α-proteobacteria, Actinobacteria, Fusobacteria, Nitrospirae and an unclassified group.

Keywords **Arctic marine sediment, bacterial diversity, culture-dependent, culture-independent**

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1 Introduction

The Arctic region has various distinct habitats for microorganisms including sea-ice, glacial ice, permafrost, tundra wetlands, oceanic water, subglacial soil, periglacial soil, and tundra soil[1]. The Arctic is being greatly altered as a result of global climate change, and the observation of microbial habitats, microbial diversity and climate impacts in Arctic Canada during the 2008 IPY field season underscored the vulnerability of polar microbial ecosystems to ongoing climate change[2].

The accumulation of organic materials in northern environments immobilizes nutrients; therefore, the Arctic is regarded as carbon sink[3]. When compared with seawater, organic matter is concentrated 10^4–10^5-fold in marine sediment and is used as substances and energy sources by microorganisms[4]. Bacteria in sediment represent a major reservoir of genetic variability similar to soil systems that show approximately 10^4 species per gram[5]. Marine sediment represents one of the most complex microbial habitats on Earth, and microorganisms in these systems contribute to bulk biomass and activity and play an important role in remineralization of organic matter[6-7].

Microbial diversity is usually investigated by culture-dependent or -independent approaches. Culture-dependent approaches are important because they enable characterization of more cultivated bacteria and their ecological roles[8]. However, only a miniscule fraction (0.1%–1%) of the bacteria can be cultivated[7]. Genetic sequences, most commonly those encoding rRNAs, provide a basis for estimating microbial phylogenetic diversity and generating taxonomic inventories of marine microbial populations[9]. Culture-independent approaches (T-RFLP, DGGE and 16S rRNA gene libraries) have been used for investigation of microbial ecology in marine sediments[8]. A few culture-independent studies of

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bacterial diversity in Arctic marine sediments have been conducted to date[4,7,10], and the sequences recovered in these studies revealed highly diverse bacterial populations, with γ-proteobacteria appearing to be one of the most significant groups in the sediments[4]. A combination of two or more approaches is likely to provide more comprehensive patterns of microbial diversity since it is likely that the limitations of one approach could be overcome by another approach[8]. However, only a few comparative investigations of bacterial diversity in marine sediments by both culture-dependent and -independent approaches have been conducted[9]. Moreover, a significant fraction of retrieved sequences in marine sediments did not belong to any known taxonomic division, indicating that there might be novel species present[11].

Biodiversity analysis of the natural bacterial community is important for understanding their ecological and biogeochemical roles in marine sediments. This study was conducted to analyze the bacterial diversity of sediment from the Arctic Ocean by both culture-dependent and -independent approaches. Specifically, the bacterial diversity of Arctic sediment and their extracellular enzymatic activities with respect to the degradation of proteins, lipids and urea were assessed, as was their ability to use various carbon sources.

2 Materials and methods

2.1 Samples

Marine sediment samples (named as SR10) with a 245-cm core length and an 11.5-cm diameter were collected during the 4th Chinese National Arctic Research Expedition on 29 August 2010 (161°00.05′W, 73°00.04′N). The depth of the sampling location was 77 m. Samples were stored at −20℃ until analysis. The bacterial diversity of the surface (0–5 cm, brown silty clay) of the sediment column was investigated.

2.2 Isolation and characterization of cultivable strains

Approximately 1 g of sediment was suspended in 5 mL Zobell 2216E medium (5 g peptone, 1 g yeast extract and 1 000 mL seawater) and subjected to shaking for 2 h at 150 rpm. The supernatant was then serially diluted, after which 50 μL was plated on Zobell 2216E agar plates and incubated at 4℃ for 7 d. The cultivable strains were subsequently isolated according to their morphological characteristics.

The growth of the cultivable strains at different temperatures (0℃–30℃) and salinities (0–120) was investigated in Zobell 2216E liquid medium based on the OD_{600} of the culture. Physicochemical characters were determined by the identification system for non-fastidious, non-enteric Gram-negative rods (API 20 NE, bioMérieux sa, France) according to the manufacturer's instructions at 4℃. Molecular identification and phylogenetic analysis were then carried out as previously described[12].

2.3 Construction of 16S rRNA gene library

Whole community DNA was extracted from approximately 0.5 g of sediment using an UntraClean soil DNA isolation kit (12800-50, MOBIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

Bacterial 16S rRNA genes of whole community DNA were amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGAC TT-3') to amplify a DNA fragment approximately 1.5 kb in length. The amplified fragment was purified using a TIAN quick Midi purification Kit (DP204-02, TianGen Biotech (Beijing) CO., Ltd., China) and then cloned into pMD18-T vector (D101A, Takara, Dalian, China) according to the manufacturer's instructions. Transformants were subsequently selected on LB agar plates supplemented with 50 $\mu g \cdot mL^{-1}$ ampicillin, X-gal and IPTG and incubated at 37℃ overnight.

2.4 Sequencing and phylogenetic analysis

The positive transformant was validated by colony PCR using the vector-targeted primers RV-M (D3880, Takara) and M13-47 (D3887, Takara). The sequenced 16S rRNA genes were then used for BLAST searching of 16S rRNA sequences to identify individual clones, which were used in construction of phylogenetic trees by the neighbor-joining method of the Mega5 software[13]. Bootstrap analysis based on 1 000 replicate datasets was performed to assess stability among clades.

2.5 Statistical analysis of the cloned library

The 16S rRNA gene sequences of clones showing ⩾ 97% sequence similarity were grouped into individual phylotypes using CodonCode Aligner[8]. The bacterial community structure was then described as described by Hill et al.[14]

2.6 16S rRNA gene accession numbers

The 16S rRNA genes of 11 cultivable strains were deposited in GenBank with accession numbers JQ586260-JQ586270, as were those of 67 uncultured phylotypes with accession numbers JQ586271-JQ586273, JQ586275-JQ586279, JQ586282, JQ586283 and JQ586286-JQ586342.

3 Results

3.1 Cultivable bacterial strains and their characterizations

Based on the distinct morphology of bacterial colonies on marine Zobell 2216E agar plates, 11 strains were isolated from Arctic marine sediment SR10 and used to construct a phylogenetic tree based on their 16S rRNA genes (Figure 1). Phylogenetic analysis indicated that these isolates belonged to three phyla (*γ*-proteobacteria, Actinobacteria and Bacteroidetes). *γ*-proteobacteria accounted for six isolates and five genera (*Shewanella, Pseudoalteromononas, Halomonas, Acinetobacter* and *Psychrobacter*). There were two Actinobacteria (*Arthrobacter* and *Microbacterium*) and three Bacteroidetes (*Olleya, Polaribacter* and *Maribacter*).

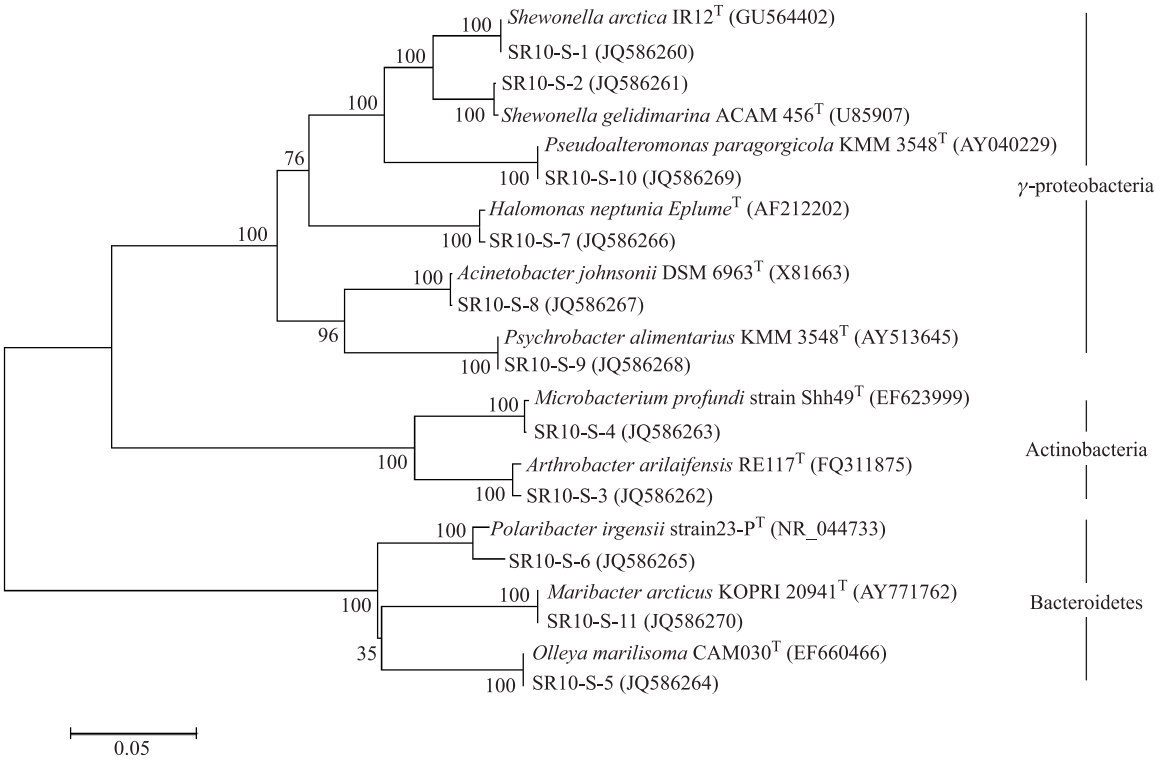

Figure 1 Neighbor-joining phylogenetic tree of 16S rRNA gene of 11 cultivable strains. Bootstrap values >50% are indicated at the nodes for 1 000 replicates. The bar represents five substitutions per 100 nucleotides.

γ-proteobacteria was the dominant phylum, accounting for 50% of cultivable strains at the genus level. The cultivable isolates had high 16S rRNA gene sequence similarity with the type strain. Indeed, the similarity of three isolates (SR10-S-1, SR10-S-10 and SR10-S-11) with their nearest phylogenetic neighbor was up to 100%, and only one isolate (SR10-S-6) had less than 99% similarity with the type strain.

The phenotypic characters of 11 cultivable strains are shown in Table 1. Based on the definition of cold-adapted microorganisms by Morita[15], strain SR10-S-1 was psychrophilic (growth at below 20°C), while the rest were psychrotrophic (growth between 0°C and 30°C). Psychrophilic strain SR10-S-1 did not require NaCl for the growth, and could grow in medium with the NaCl concentration of 60 g·L^{-1}. This strain secreted various extracellular hydrolases, including protease, lipase, galactosidase and glucosidase, and could use mannitol, maltose and malic acid as a carbon source for growth. With the exception of strain SR10-S-10, these isolates did not require NaCl for the growth, and most could grow in medium with 90 g·L^{-1} NaCl. These isolates secreted at least one type of extracellular hydrolytic enzyme (protease, lipase, urease, galactosidase or glucosidase) at 4°C, and used at least three of the 12 tested carbon sources (Table 1). Notably, SR10-S-5 produced four types of extracellular hydrolytic enzymes and assimilated nine kinds of carbon sources.

3.2 Statistical analysis of 16S rRNA gene library

A total of 142 clones with an approximate insert size of 1.5 kb were selected for sequencing. According to Shivaji et al.[8] and Zeng et al.[4], clones can be grouped into one group (phylotype) when the 16S rRNA gene sequence similarity is $\geqslant$ 97%. Based on BLAST analysis and assembly using the Codon Code aligner software, 67 phylotypes were identified. The library coverage *(C)* of the 16S rRNA gene library constructed in this study was 0.68, suggesting that nearly one third of species diversity in the sediment was still undiscovered. The high Simpson index (0.96) indicated that there was a dominant phylotype in the sediment, which was confirmed by the subsequent phylogenetic analysis of the 16S rRNA gene library (Table 2).

3.3 Phylogenetic analysis of 16S rRNA gene clone library

Similarity analysis of the 16S rRNA genes indicated that most of the 142 clones showed the highest similarity with uncultured bacterial clones. Phylogenetic analysis of 67 phylotypes clustered with their nearest type strains is shown in Figure 2 (clones belonging to Proteobacteria) and Figure 3 (all clones except those belonging to Proteobacteria). The 67 phylotypes fell into nine major lineages of the domain bacteria, including α-, γ-, δ-, ε-proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria, Nitrospirae and an unclassified group.

Among all clones obtained, 83.1% were affiliated with Proteobacteria, which contained 52 phylotypes and accounted for 77.6% of the total phylotypes. The γ-proteobacteria was

Table 1 Phenotypic characteristics of 11 cultivable strains isolated from Arctic sediment

Strains	Growth characteristics		Extracellular hydrolase	Carbon source utilization
	Temperature range/°C	NaCl tolerance /(g·L^{-1})		
SR10-S-1	0–20	0–60	protease, lipase, galactosidase, glucosidase	mannitol, maltose, malic acid
SR10-S-2	0–30	0–90	lipase, urease, protease	glucose, arabinose, maltose, glyconate, malic acid, phenylacetic acid, sodium citrate
SR10-S-3	0–30	0–90	urease	glucose, arabinose, maltose, glyconate, malate, phenylacetic acid, sodium citrate
SR10-S-4	0–30	0–60	lipase, glucosidase, galactosidase	glucose, arabinose, mannitol, maltose, glyconate, malic acid
SR10-S-5	0–30	0–60	urease, glucosidase, galactosidase, protease	glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, glyconate, malic acid, sodium citrate
SR10-S-6	0–30	0–90	lipase, arginine, dextrose, galactosidase	arabinose, maltose, glyconate, malic acid, sodium citrate
SR10-S-7	0–30	0–90	protease, lipase	glucose, arabinose, maltose, glyconate, malic acid, sodium citrate, phenylacetic acid
SR10-S-8	0–30	0–30	lipase	capric acid, malic acid, citrate
SR10-S-9	0–30	0–90	urease, lipase	glucose, arabinose, maltose, malic acid, sodium citrate, phenylacetic acid
SR10-S-10	0–30	30–90	protease, lipase	mannitol, maltose, glyconate
SR10-S-11	0–30	0–90	lipase, urease	glucose, arabinose, maltose, glyconate, malic acid, sodium citrate, phenylacetic acid

Table 2 Statistical analysis of 16S rRNA gene library

Clones	Phylotypes	Coverage (C)	Shannon-Wiener index (H')	Simpson index (D)	Evenness (E)	Richness (d_{Ma})
142	72	0.68	5.37	0.96	0.89	9.23

the predominant sub-phylum, including 19 phylotypes and 52 clones. δ-proteobacteria was the second-most dominant sub-phylum following γ-proteobacteria, including 17 phylotypes and 31 clones (Figure 2). ε-proteobacteria was the third dominant sub-phylum, accounting for 16.9% of the total clones. Nitrospirae and Fusobacteria included only one phylotype and one and two clones, respectively. At the level of the phylotype, SR10-128 was the most dominant. Specifically, 16 clones were affiliated with this phylotype, accounting for 11.3% of the total clones (Figure 3). These results were consistent with the high Simpson index of the 16S rRNA gene library in that they indicated a dominant phylotype in the sediment.

Analysis of the 16S rRNA gene library in this study also showed that a small fraction of retrieved sequences (6.0% of total phylotypes and 2.8% of total clones) were difficult to classify into any known taxonomic division. Phylotypes SR10-80, SR10-95, SR10-43 and SR10-200 were novel phylogenetic groups, which indicated that there are novel species in Arctic sediment.

4 Discussion

Bacteria comprise the bulk of overall carbon biomass and associated biogeochemical cycling in sediments within aquatic ecosystems, where they play significant ecological and biogeochemical roles[16]. Bacterial species are usually described by empirical criteria. Hagström et al.[17-18] analyzed the degree of DNA-DNA relatedness versus 16S rDNA similarity for a high number of marine isolates and found that a 16S rDNA sequence similarity of $\geqslant$97% was a reasonable level for grouping bacteria into species. The species definition based on similarity has matured to the point that

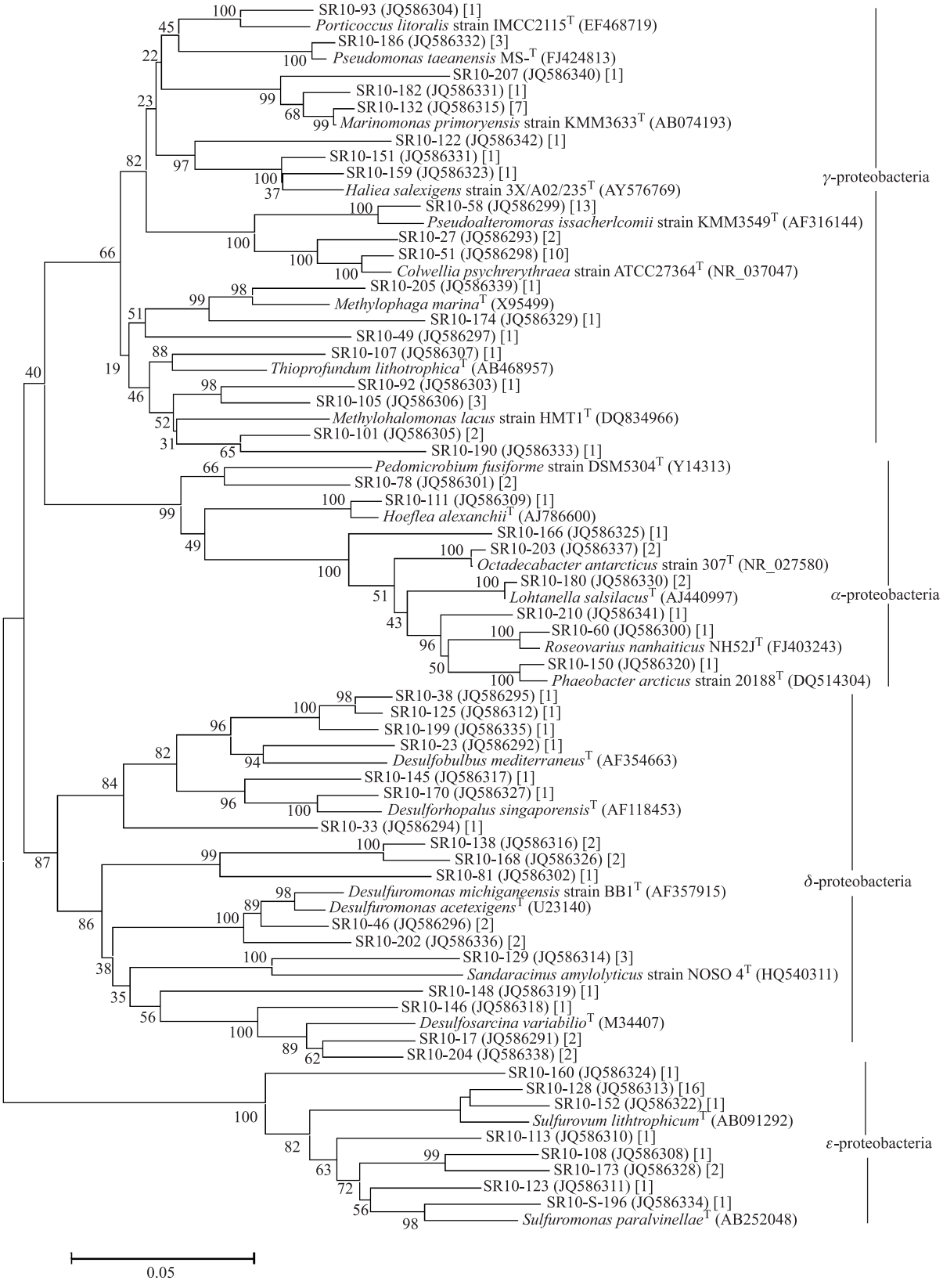

Figure 2 Neighbor-joining phylogenetic tree of 16S rRNA gene clones from the library. Only clones belonging to Proteobacteria are included in this tree. Numbers in brackets following the clone accession number indicate the number of times it was found in the clone library of sediment SR10. Bootstrap values >50% are indicated at the nodes for 1 000 replicates. The bar represents five substitutions per 100 nucleotides.

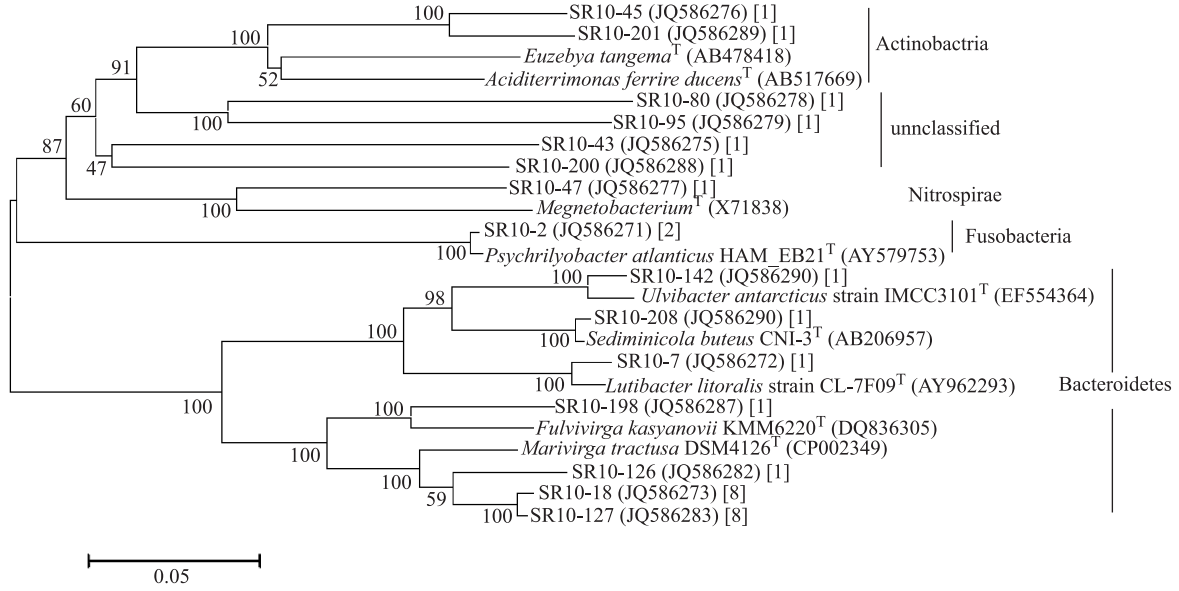

Figure 3 Neighbor-joining phylogenetic tree of 16S rRNA gene clones from the library. All clones except those belonging to Proteobacteria are included in this tree. Numbers in brackets following the clone accession number indicate the number of times it was found in the clone library of sediment SR10. Bootstrap values >50% are indicated at the nodes for 1 000 replicates. The bar represents five substitutions per 100 nucleotides.

it has been entered into a major microbiological textbook[17]. Using this criterion, 67 phylotypes were identified from 142 clones (Table 2). According to the criterion that higher than 93% similarity indicates a group at the genus level[4], 49 phylotypes were identified. These results further indicate that there might be abundant and diverse bacterial populations in Arctic marine sediments.

Using the cultivable approach, 11 isolates were identified based on their distinct colony morphology on 2216E agar plates. Since these strains were isolated from Arctic marine sediment, it is not surprising that they were exclusively psychrotrophic or psychrophilic. Because of their ability to produce extracellular cold active hydrolase and assimilate various carbon sources, these isolates might play an important role in the transformation of complex organic compounds[8].

In this study, clones related to Proteobacteria accounted for 83.1% of the total clones. Consistent with previous investigations of marine sediments, γ- and δ-proteobacteria are the dominant groups[4, 19-20]. Notably, no sequences affiliated with β-proteobacteria were detected in this study, even though they were identified in the 16S rRNA gene library of Arctic sediments constructed by Zeng et al.[4], Tian et al.[7] and Li et al.[10]

Three clones (2.1% of total clones) affiliated with Actinobacteria were grouped into two phylotypes. Previous studies revealed that Actinobacteria accounted for a small proportion, but played a significant role and were ubiquitous in marine microbial communities[21]. Bacteroidetes, which consisted of eight phylotypes with 14 clones, accounted for 9.9% of the total clones. These organisms are widely distributed in marine environments and play an important role in carbon cycling owing to their ability to degrade particulate organic matter in the ocean[4].

Previous studies indicated that the rates of sulfate reduction and benthic carbon mineralization in Arctic sediments were comparable to those in temperate or even tropical sediments[22-23]. Sulfate reducer phylotypes belonging to δ-proteobacteria have been reported to be dominant in Arctic sediments of Spitsbergen[4,20]. Many clones affiliated with sulfur-metabolizing bacteria were also detected in the present study. Phylotypes affiliated with δ-proteobacteria were the second-most abundant in the 16S rRNA gene library, most of which belonged to sulfate-reducing bacteria, including *Desulforhopalus*[24], *Desulfobulbus*[25], *Desulfuromonas*[26] and *Desulfosarcina*[27]. Moreover, three phylotypes (SR10-128, SR10-152 and SR10-196) belonging to ε-proteobacteria were affiliated with sulfur-oxidizing genera of *Sulfurovum*[28] and *Sulfuromonas*[29]. These results indicated that bacteria might play a significant role in sulfur cycles in Arctic marine sediments.

Early studies describing the microbial diversity of marine sediment communities relied on the isolation, identification and characterization of cultivable cells, which might only represent $\leqslant$ 0.1%–1% of the total microbial community[30]. Culture-independent methods based on extraction and analysis of total nucleic acids can avoid the shortcomings of isolating cultivable microorganisms and theoretically enable identification of the entire microbial population[4,31]. In addition to PCR-based analysis of the 16S rRNA gene, quantitative methods such as fluorescence in situ hybridization (FISH) and rRNA slot blot hybridization

have also been adopted to investigate the major phylogenetic groups in marine sediments. The phylogenetic composition of marine Arctic sediment (Svalbard) was investigated by FISH and rRNA slot blot hybridization with 16S rRNA-targeted oligonucleotide probes[32]. In this study, only 11 isolates with distinct morphological characters on Zobell 2216E agar plates were isolated. These isolates were affiliated with three phyla, γ-proteobacteria, Actinobacteria and Bacteroidetes. However, a total of 67 phylotypes were identified among 142 clones using the 16S rRNA gene library, and these were grouped into nine major lineages of the Bacteria.

In summary, higher bacterial diversity, including nine bacterial divisions (67 phylotypes), was observed in surface sediments. *γ*-, *δ*-, and *ε*-proteobacteria were the three dominant divisions. The three phyla (*γ*-proteobacteria, Actinobacteria and Bacteroidetes) detected by culture-dependent methods were all detected in the 16S rRNA gene library. At the phylotype level, 67 phylotypes were detected in the 16S rRNA gene library, which was much more than that of the cultivable isolates (11 phylotypes). Notably, the counterpart of only one cultivable phylotype (*Pseudoalteromonas*) was detectable in the 142 clones sequenced, possibly because of lower clone coverage (*C*) and because more clones should be sequenced. When compared with earlier studies[4,7], the results of this study also indicated that bacteria affiliated with Proteobacteria were dominant in the marine sediments of the Arctic. Moreover, as a distinct habitat, Arctic sediment was a rich source of bacteria and novel species with considerable exploitation potential.

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