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Characterization of *Cytophaga-Flavobacteria* Community Structure in the Bering Sea by Cluster-specific 16S rRNA Gene Amplification Analysis

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A newly designed *Cytophaga-Flavobacteria*-specific 16S rRNA gene primer pair was employed to investigate the CF community structure in the Bering Sea, revealing a previously unknown and unexpected high CF diversity in this high latitude cold sea. In total, 56 clones were sequenced and 50 unique CF 16 rRNA gene fragments were obtained, clustering into 16 CF subgroups, including nine cosmopolitan subgroups, five psychrophilic subgroups, and two putatively autochthonous subgroups. The majority of sequences (82%) were closely related to uncultured CF species and could not be classified into known CF genera, indicating the presence of a large number of so-far uncultivated CF species in the Bering Sea.

Keywords: *Cytophaga-Flavobacteria*, 16S rRNA gene, community structure, diversity, the Bering Sea

Among the heterotrophic bacteria in the ocean, the two most abundant groups are *Proteobacteria* and a subgroup of bacteria in the *Bacteroidetes* division, frequently called *Cytophaga-Flavobacteria* (CF) cluster [15]. In oceanic habitats, this cluster could account for approximately 30% of total cells in temperate coastal waters [9], up to 30% of total cells in North Sea surface waters [10, 12], and up to 72% of the total bacterioplankton in Antarctic surface waters [12], and thus plays an important role in marine ecosystems with respect to the biogeochemical cycling of biogenic elements [15].

However, only a few studies concerning CF community structure have been reported in marine environments; for example, in the Delaware Estuary [16], the near shore of Plymouth [19], and the Southern Ocean [1, 2]. To understand more about the CF biogeography and diversity pattern in marine ecosystems, more representative marine regions need to be explored. Among the global oceans, the Bering Sea is one of the most productive seas, and little is known concerning CF diversity there. The prosperous phytoplankton throughout the year in the Bering Sea provided abundant organic matter for bacterioplankton, especially for the CF cluster, which was reported to be especially proficient in degrading various high-molecular-weight (HMW) dissolved organic matter (DOM) such as cellulose, chitin, and pectin [15]. How CF were distributed in such a mesotrophic, high latitude, and cold sea is an interesting topic. The results could provide us with more understanding of the CF distribution pattern in global oceans. In a previous work, we designed a specific primer pair for the CF cluster and confirmed the improved amplification capacity by detailed computer analysis [7]. In this paper, we further used this primer pair to characterize the CF community structure in the Bering Sea.

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Analysis of CF 16S rRNA Gene Sequences from the Bering Sea

Surface seawater samples were collected from sampling station BS-E (54.99°N, 171.82°E; July, 2003). Water temperature and salinity, measured using the sampling apparatus (SBE 9/11 plus CTD system, SeaBird Inc., U.S.A.), were 10.5°C and 32.9 psu, respectively. Biomass for DNA extraction was collected by filtering 2 l of seawater onto a 0.22-μm polycarbonate filter (Millipore, U.S.A.). DNA was extracted using the hot SDS, phenol: chloroform:isoamyl alcohol, ethanol precipitation extraction protocol as described initially by Fuhrman *et al.* [11] and modified by Zeng *et al.* [23].

CF 16S rRNA gene clone libraries were constructed through PCR using a newly designed CF-specific primer pair (CF315-F, 5'-ACKGGYACTGAGAYACGG-3'; CF967-R, 5'-GGTAAGGTTCCTCGCGTA-3'). The 50-μl PCR reaction mixture consisted of 0.15 μM each primer, 0.4 mM each dNTP, 5 μl of 10× PCR buffer, 1.5 mM $MgCl_2$, 1 unit *LA*-Taq DNA polymerase (TaKaRa Co., Dalian, China), and 2 μl of DNA solution. The reaction was performed on a T3 thermocycler (Biometra Co., Germany) with a hot

start and touchdown PCR program: denaturing for 5 min at 94°C; pause at 80°C for adding DNA polymerase to avoid nonspecific starting; 30 cycles of denaturing for 1 min at 94°C, annealing for 1 min at a temperature decreasing from 60°C to 55°C in increments of −0.5°C per cycle and then at 55°C for 20 cycles, and extension for 1 min at 72°C; final extension for 10 min at 72°C. PCR products were gel-purified, ligated into pMD18-T vector (TaKaRa, Dalian, China), and then transformed into competent *E. coli* DH5α (TaKaRa, Dalian, China). fifty-six clones were randomly selected for sequencing (Sangon Inc., Shanghai, China) on an ABI 370 genetic analyzer (Applied Biosystem, U.S.A.) using the upstream primer CF315-F as the sequencing primer. The GenBank accession numbers of the CF 16S rRNA gene fragments (ca. 600 bp) reported in this paper are DQ656121-DQ656170.

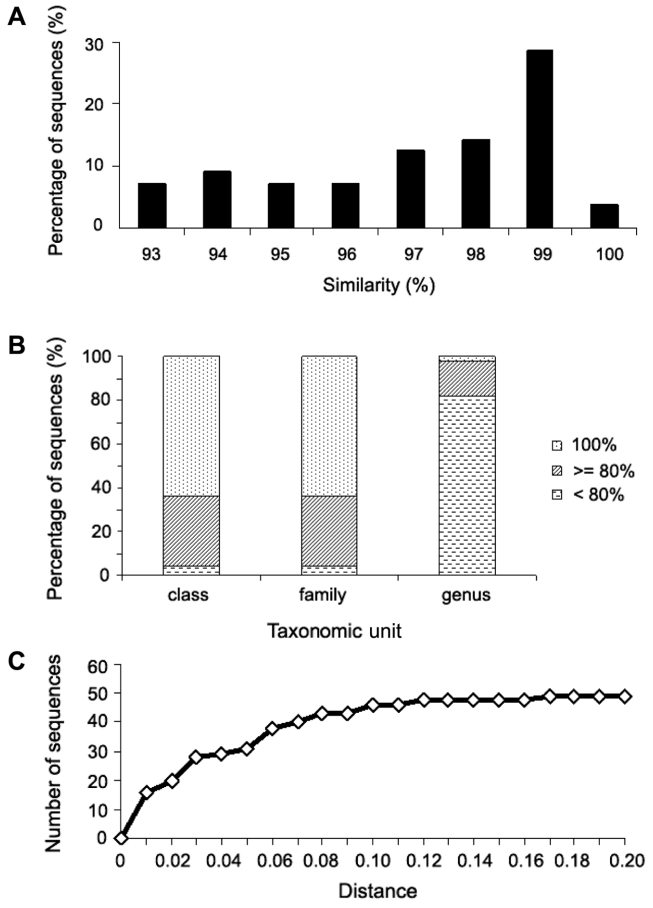

Fig. 1. Statistical analysis of the Bering Sea CF 16S rRNA gene sequences.
Distribution of BLAST similarity value (**A**), RDP classifier statistics (**B**), and DOTUR clustering analysis (**C**). The BLAST similarity values were retrieved from the results of BLAST hits in the GenBank database; the RDP classifier data were from the RDP online Classifier tool's result; Clustering data were obtained using a newly developed computer program, DOTUR, for defining operational taxonomic units and estimating the species richness.

Chimeric artifact analysis by the Check-Chimera program [8] found no abnormal sequence. Taxonomy analysis by the RDP classifier tool [8] showed that all sequences were distinctly affiliated with the CF cluster. As a comparison, a preamplification analysis of the same DNA sample was performed using the only published CF specific primer pair CF316 and EubA-R [16]. The preliminary results showed that no more than 40% of total 50 clones were affiliated with the CF cluster, represented by nine distinct sequences, as shown in Fig. 3. These results indicated that the newly designed primer pair (CF315-F and CF967-R) was highly specific and efficient in retrieving CF 16S rRNA gene fragments from natural marine environments.

To assess how many sequences could be classified into known CF genera, we performed a classifier analysis using the Classifier Tool in the Ribosomal Database Project Web site (RDP; http://rdp.cme.msu.edu/classifier/classifier.jsp) [8]. The result indicated that only nine CF sequences (18%) could be classified into a known genus with a more than 80% confidence value (Fig. 1B). Furthermore, to assess the degree of how these new CF sequences were represented by those that have been deposited in the public database, BLAST homogeneous analysis (http://www.ncbi.nih.gov/blast) was performed for each sequence. The results showed that the similarity value with GenBank records ranged from 93%–100%, with 82% of the sequences having more than 95% similarity (Fig. 1A). This indicated that a large number of as yet uncultivated CF species were dwelling in this cold sea. The overall CF diversity was also analyzed. The sequence clustering and rarefaction analysis were performed using

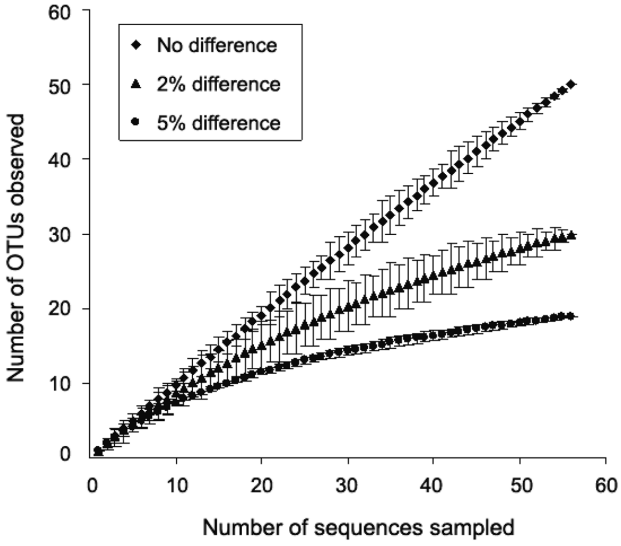

Fig. 2. Rarefaction curve of the Bering Sea CF 16S rRNA gene clone library.
Error bars represent 95% confidence interval (CI).

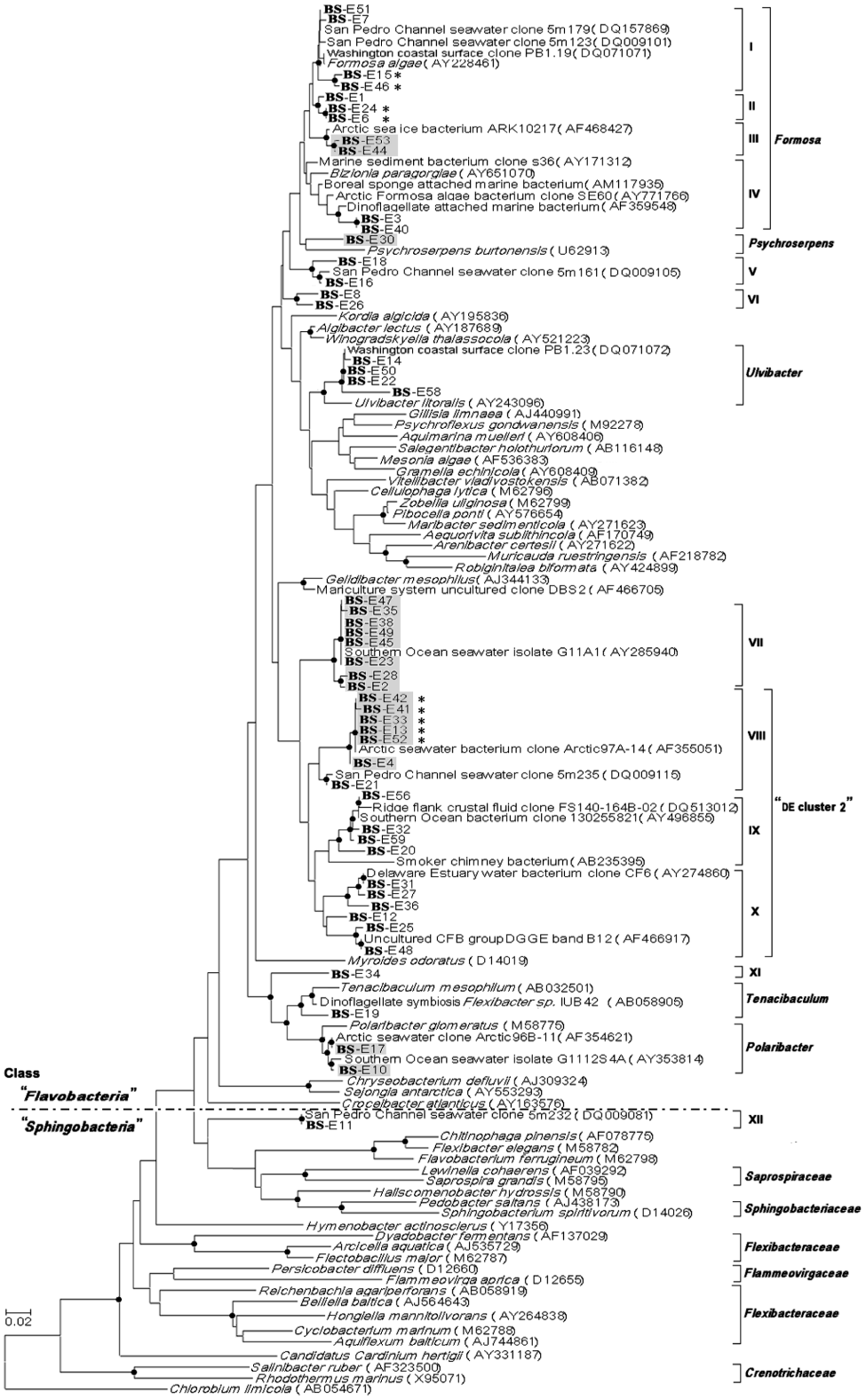

Fig. 3. Phylogenetic analysis of the Bering Sea CF 16S rRNA gene sequences (Station BS-E).
The phylogenetic tree was constructed using the neighbor-joining algorithm. Bootstrap values were obtained with 1,000 resamplings. The nodes with a bootstrap value of more than 50% are shown with a solid black dot. The 16S rRNA gene sequence of *Chlorobium limicola* was used as the outgroup. The scale bar represents 2% nucleotide substitution. Putative psychrophilic taxa are shown in grey. The sequences that appeared in the preamplification analysis using the primer pair CF316 and 1492R are marked with an asterisk.

a newly developed computer program for Defining Operational Taxonomic Units and Estimating Species Richness (DOTUR) [20]. Employing 2% and 5% nucleotide differences as OTU definition criteria for species and genera [20], 20 and 31 CF sequences was clustered into seperate OTUs, respectively (Fig. 1C), meaning that 30 species or 19 genera were present in the sample. Considering the limited sampling effort in the clone library, this result suggested a high diversity of CF species and genera present in the Bering Sea. In addition, the assessment of within-sequence distance by the DOTUR program revealed that the highest distance within two sequences reached a 17% nucleotide difference (Fig. 1C), which indicated that quite different CF species were sharing the same niche in the Bering Sea. As an indication of sampling effort, the unsaturated rarefaction curves further revealed that a part of the CF diversity was unsampled (Fig. 2).

Characterization of CF Community Structure in the Bering Sea

The nearest neighbors from the GenBank database through BLAST search and representative sequences of each CF genera from the RDP database [8] served as reference sequences for the phylogenetic tree inference. Sequences were aligned using the ClustalX 1.80 program [22]. The neighbor-joining tree was constructed using the MEGA 3.0 program [17] from the distance matrix corrected with Kimura's two-parameter model 2.0 [14]. Phylogenetic analysis revealed that all CF sequences were clustered into 16 subgroups (Fig. 3) and all sequences were affiliated with the class *Flavobacteria*, except for BS-E11, which belonged to the class *Sphingobacteria*.

The BS-E station was located near the central North Pacific and far away from the coast, featured by cold water and open ocean properties. Phylogenetic analysis revealed that nearly all CF sequences were clustered with sequences from marine environments, consistent with these geographical features of the Bering Sea. Twelve sequences (Fig. 3, in grey) were closely related to CF sequences from cold polar sea waters. These clusters (BS Clusters III, VII, and VIII, *Polaribacter*) appeared to be restricted to colder waters. In addition, the genera *Polaribacter* and *Psychroserpens*, with which the clones BS-E10, BS-E17, and BS-E30 were affiliated, contain exclusively psychrophilic species [5, 13]. Therefore, these five clusters possibly represented psychrophilic CF subgroups that could adapt well to cold Bering Sea waters. Water temperature has been revealed previously to be an important physical barrier in the ocean, especially in polar and adjacent seas [2], affecting microbial community distribution [18, 21]. Abell and Bowman [2] suggested that temperature selection plays an important role in shaping the microbial community by selecting for psychrophilic species. Isolation of autochthonous psychrophilic CF species has also been reported in Antarctic waters and sea ice [5, 6, 13]. Here, we found five psychrophilic subgroups in the sample from the Bering Sea. This result was consistent with the cold and higher productivity feature of the Bering Sea throughout the year. Moreover, the finding of many psychrophilic subgroups in one sample also benefited from the using of more specific CF primers. However, the lack of physiological data made us unable to assess the detailed response of psychrophilic subgroups to temperature and nutrient. Field experiments on the RNA level need to be carried out.

The majority of CF subgroups (*Formosa*, *Psychroserpens*, BS Clusters V, VIII, IX, X, and XI, *Ulvibacter*, and BS-E11) were also found in temperate or subtropic seas (Fig. 3). The subgroups VIII, IX, and X can be combined into a larger cluster, equivalent to a clade, which is designated the DE cluster 2 [16] and named for clones from the Delaware Estuary, and the Chukchi Sea, Arctic Ocean [2]. DE Cluster 2 was widely distributed in temperate to polar waters, such as the coast of England [19], the North Sea [24], the U.S. west coast [4], and the Arctic Ocean [3], as previously revealed [2, 16]. These results indicated that a large number of the CF species in the Bering Sea might be widely dispersed in global marine environments and could adapt to a wide range of temperatures. The two subgroups VI and XII did not cluster with the nearest neighbors in the database and were distributed separately in the phylogenetic tree. They might be the autochthonous CF species in the Bering Sea.

In summary, this is the first report concerning the CF community structure in the Bering Sea. A previously unknown and unexpected high CF diversity was revealed. Psychrophilic, cosmopolitan, and autochthonous species dominated the CF community. Specific CF primers were shown to be helpful for understanding the marine CF community structure, especially the composition and biogeography of its subgroups. This study may also indicate an interesting future research topic: the biogeography of the CF cluster and its subgroups in the ocean.

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