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# Some Universal Characteristics of Intertidal Bacterial Diversity as Revealed by 16S rRNA Gene-Based PCR Clone Analysis

**SHUANG, J. L., C. H. LIU*, S. Q. AN, Y. XING, G. Q. ZHENG, AND Y. F. SHEN**

*State Key Laboratory of Pharmaceutical Biotechnology, State Key Laboratory of Pollution Control and Resource Reuse, Nanjing University, Nanjing 210093, China*

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**Abstract** A 16S rDNA clone library was generated to investigate the bacterial diversity in intertidal sediment from the coast of the Yellow Sea, P. R. China. A total of 102 clones were sequenced and grouped into 73 OTUs using a phylogenetic approach. The sequenced clones fell into 11 bacterial lineages: *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Spirochaetes*, and candidate divisions of BRC1, OP3, and OP11. Based on a phylogenetic analysis of these bacteria, together with the ten most closely related sequences deposited in the GenBank, it was concluded that intertidal bacteria are most likely derived from marine bacteria with a remarkable diversity, and some are particularly abundant in intertidal sediment.

**Key words:** Bacterial diversity, 16S rDNA sequence analysis, intertidal sediment

The bacteria in intertidal sediment play an important role in the mineralization of organic matter and degradation of pollutants, resulting in many recent reports. However, most previous studies of these bacteria have focused on specific functional groups, such as SRB [3, 6, 12], as the processes they catalyze can be measured directly. Thus, the characteristics of the bacterial diversity in intertidal sediment have received relatively little attention [4, 11].

It is well known that the PCR amplification of 16S rRNA genes followed by cloning and sequencing is a powerful means of investigating soil bacterial diversity, since it allows the screening of both cultured and uncultured bacteria. Accordingly, this study used this method to investigate the bacterial diversity in intertidal sediment from China, and to disclose some common characteristics of bacteria inhabiting the intertidal ecosystem.

*Corresponding author
Phone: 86-25-83685469; Fax: 86-25-83592705;
E-mail: chliu@nju.edu.cn

## MATERIALS AND METHODS

### Sampling

Ten intertidal sediments were collected from a site at Dafeng (120°6–7'N, 33°3–7'E) on the coast of the Yellow Sea on April 6, 2004, using a soil corer that was hammered into the soil to a depth of 10 cm. The samples were placed in polycarbonate bags, held on ice during transport to the laboratory, and then stored at -80°C for 16S rDNA analysis.

### Extraction of Total DNA

The total DNA from 1 g of an equal mixture of the ten sediment samples was extracted using a modified method [9, 32, 35]. Briefly, the sample mixture was incubated with 3 ml of a DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl, pH8.0, 100 mM EDTA, 4% sodium dodecyl sulfate (SDS), 2% hexadecyltrimethylammonium bromide (CTAB), 0.5% 2-mercaptoethanol], 100 mg of RNase, and 0.1 ml of lysozyme (10 mg/ml) at 37°C for 30 min. Then, 0.3 ml of proteinase K (10 mg/ml) was added and the mixture incubated at 65°C for an additional 30 min. The cell lysis efficiency of the DNA extraction procedure was checked by counting the DAPI-stained cells in aliquots. Thereafter, the DNA was further extracted with phenol (pH 8.0), phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). The crude DNA was then precipitated with isopropanol and further purified with a DNA Gel Purification Kit (Tianwei, Inc) following the protocol of the manufacturer. The purified DNA was finally quantified by agarose gel electrophoresis.

### PCR Amplifications

The primers used for amplification of the bacterial 16S rDNA were 8f (5'-AGAGTTTGATCMTGGC-3'; positions 8–23 of *Escherichia coli* numbering) and 1542r (5'-AAAGGAGGTGATCCA-3'; positions 1556–1542) [25]. The PCR mixtures were prepared in 50 μl volumes, containing 0.5 μM of each primer, 200 mM of each deoxyribonucleoside triphosphate, 5 μl of the 10×PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl; pH 8.3), 1 U of Taq DNA polymerase (Tianwei Inc.), and 1 μl of the extracted DNA. The DNA amplification was performed in a GeneAmp PCR system 2400 (Perkin Elmer) with an initial denaturation for 2 min at 94°C, followed by 25 cycles of denaturation (0.5 min at 94°C), annealing (1 min at 55°C), and extension (1 min at 72°C), plus a final extension for 10 min at 72°C to facilitate the TA cloning. The PCR products were further purified using a PCR Purification Kit (Tianwei Inc.) and confirmed by horizontal electrophoresis on a 1% agarose gel in a TAE buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA).

### Cloning and Sequencing

Ten μl of the amplified DNA was cloned into a plasmid vector pG-T from a TA cloning kit (Tianwei, Inc.) and 5 μl of the ligation products subsequently transformed into competent *E. coli* JM109. The cells were then grown in a Luria-Bertani agar medium at 37°C for 24 h. Blue-white selection was used to screen the clones containing the correct length inserts (approx. 1.5 kb). A total of 102 positive clones were chosen randomly for PCR amplification with the vector-specific primers M13, according to the manufacturer's instructions (Tianwei, Inc.). The PCR products were purified using a PCR Purification Kit (Tianwei, Inc.), and then sequenced with a bacteria-specific primer 8f using an ABI 3100-Avant Genetic Analyzer (Applied Biosystems). The sequencing reactions were carried out by cycle sequencing using a BigDye Terminator (Applied Biosystems) and the 8f primer.

### Sequence Analysis

The possible chimeras were analyzed using the CHECK_CHIMERA program from the ribosomal database project (RDP) and BELLEROPHON program (http://foo.maths.uq.edu.au/~huber/bellerophon.pl). The clones that were randomly selected for sequencing were considered to belong to the same operational taxonomic unit (OTU) if they were ≥99% identical over the sequenced region of the 16S rRNA gene [1]. The ten most closely related 16S rDNA sequences for each nonchimeric sequence were retrieved from DNA databases using the BLAST program (http://www.ncbi.nlm.nih.gov), whereas the type sequences were obtained from the RDP. All the sequences were aligned using the ClustalW program (ver. 1.60) [31] and realigned manually. A neighbor-joining (NJ) analysis was also performed using the NEIGHBOR program [26], and a phylogenetic tree constructed from a distance matrix based on the evolutionary distances (Jukes-Cantor) calculated using the PHYLIP package 3.63 program [5]. The bootstrap confidence values were obtained from 1,000 replicates. Sequences from the libraries for Sapelo Island in the USA and Guanghwa Island in Korea were retrieved from the GenBank, and realigned using the ClustalW program. The significant differences between these libraries were determined using the LIBSHUFF program [29].

### Nucleotide Sequence Data

The sequences determined in this study have been deposited in the GenBank nucleotide sequence database under accession numbers DQ167024 through DQ167125.

## RESULTS

The analysis of DNA from microbial populations in soil and sediment samples is fraught with difficulties, as the direct lysis of cells within the soil matrix often results in the coextraction of other soil components, including potent organic inhibitors, such as humic and fulvic acids. These components can then prevent the PCR amplification of DNA. Therefore, this study applied a modified procedure, involving the addition of chemicals (SDS, CTAB, and EDTA) and enzymes (lysozyme and proteinase K) to the extraction buffer and the incorporation of an inhibitor removal gel-purification step. As a result, more than 95%

**Table 1.** Phylogenetic differences between bacteria from Guanghwa Island and bacteria in the present study.

| Phylum | Guanghwa island[a] | Present study |
|---|---|---|
| *Bacteroidetes* | 10.8% | 21.6% |
| *Alfaproteobacteria* | 12.7% | - |
| *Betaproteobacteria* | 1.0% | 4.9% |
| *Gammaproteobacteria* | 27.5% | 13.7% |
| *Deltaproteobacteria* | 19.6% | 14.7% |
| *Epsilonproteobacteria* | 2.0% | 8.8% |
| *Planctomycetes* | 2.0% | 10.8% |
| *Chloroflexi* | 7.8% | 11.8% |
| *Acidobacteria* | 4.9% | 2.0% |
| *Actinobacteria* | 2.9% | 2.9% |
| *Verrucomicrobia* | 2.0% | - |
| *Firmicutes* | - | 3.9% |
| *Spirochaetes* | - | 1.0% |
| BRC1 | 1.0% | 1.0% |
| OP3 | - | 2.0% |
| OP11 | 1.0% | 1.0% |
| KSB1 | 1.0% | - |
| WS3 | 2.0% | - |
| Unclassified bacteria | 2.0% | - |

[a]Data obtained from paper by Kim *et al.* [11].

of the bacterial cells from the intertidal sediment were lysed, and the DNA (MW >20 kb) recovery was over 30 μg/g soil.

## Similarity with Database Sequences

The PCR products of the 102 randomly selected clones constructed from the intertidal sediment were partially sequenced (most were 600–700 bp) using the bacterial 16S rDNA-specific primer 8f. Based on an identical criterion of ≥99%, all the sequences were grouped into 73 OTUs, except for one putative chimera that was omitted from this study. The Shannon diversity index was 4.2, and a statistical analysis showed that the bacterial diversity in this study was significantly different from that in other intertidal areas, such as Sapelo Island, USA and Guanghwa Island, Korea ($P<0.001$). Table 1 shows the phylogenetic difference between the bacteria obtained from Guanghwa Island and the bacteria in this study.

An analysis of the 618 closest 16S rDNA sequences retrieved from DNA databases using the BLAST program indicated that 29% of them were mainly distributed in intertidal or estuary environments, 23% in marine locations,

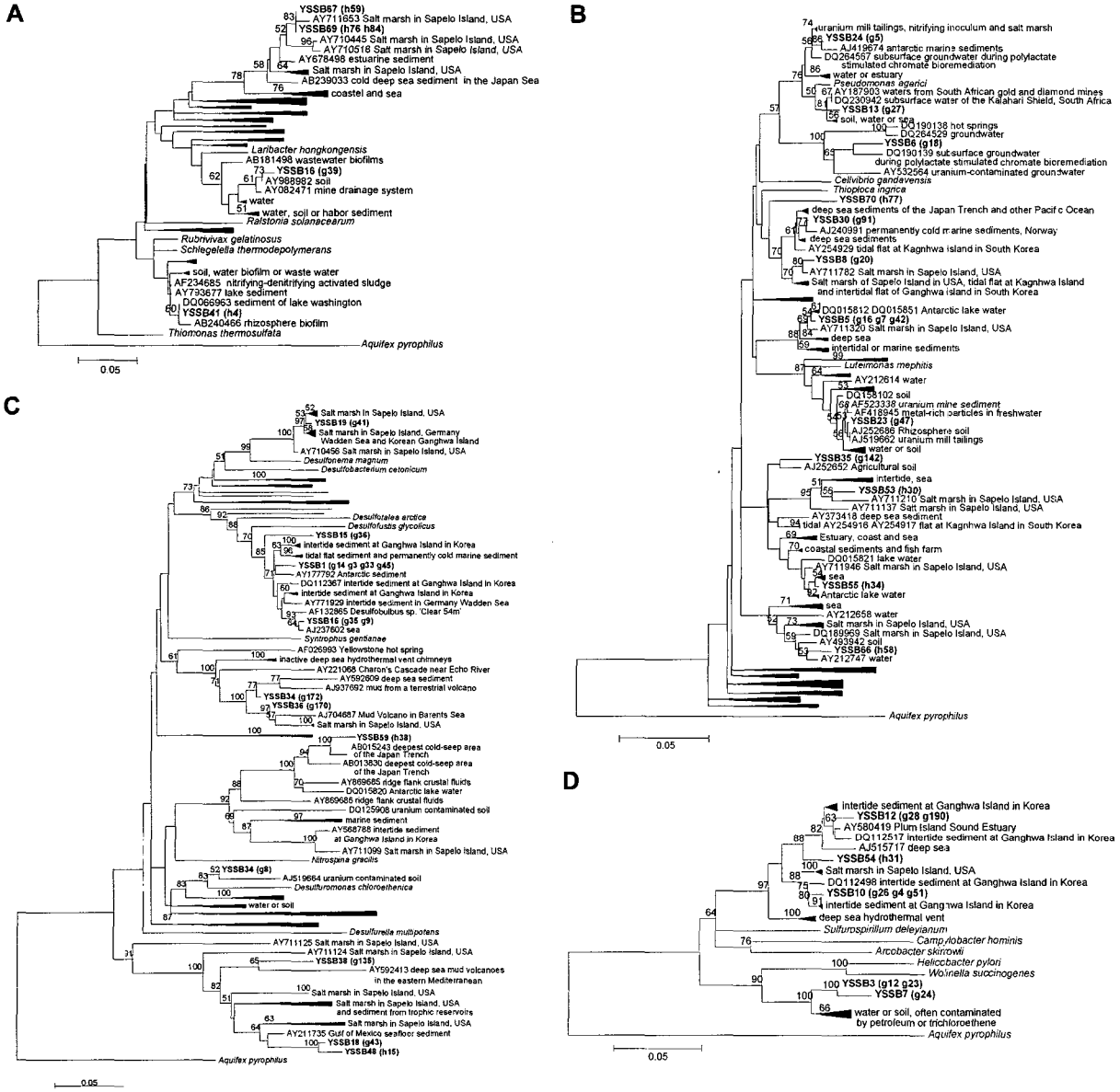


**Fig. 1.** Phylogenetic trees constructed using the neighboring-joining method based on 16S rDNA sequences of proteobacterial clones from intertidal sediment. *Betaproteobacteria* (**A**), *Gammaproteobacteria* (**B**), *Deltaproteobacteria* (**C**), and *Epsilonproteobacteria* (**D**). *Aquifex pyrophilus* was used as the outgroup. The scale bar represents the mean number of nucleotide substitutions per site. Bootstrap values (1,000 resamplings) are shown for frequencies at or above a threshold of 50%. Each OTU in this study is indicated in bold font letters, and its clones are shown in brackets. The relative sequences show their GenBank Accession number and locations. A cuneiform indicates a compressed branch.

19% in fresh water, and 13% in continental soil. Moreover, the majority of the retrieved sequences (about 87%) were from uncultured clones. Of the sequences obtained in this study, 14% showed a high similarity (>97%) with known bacterial sequences deposited in the GenBank, 57% a medium similarity (93–97%), and 29% a weak similarity (<93%).

**Phylogenetic Analysis**

A phylogenetic tree was constructed using an alignment analysis of the intertidal DNA sequences grouped into 73 OTUs. As a result, 11 phylogenetic bacterial lineages were recovered from the intertidal sediment: *Proteobacteria* (32), *Bacteroidetes* (13), *Chloroflexi* (10), *Planctomycetes* (9), *Acidobacteria* (2), *Firmicutes* (2), *Actinobacteria* (1), *Spirochaetes* (1), and uncultured candidate divisions BRC1 (1), OP3 (1), and OP11 (1). Among them, *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Chloroflexi* were clearly the major components of the intertidal sediment (87% of total OUTs), and particularly *Proteobacteria*, which accounted for 43.8% of the total OTUs.

***Proteobacteria***

The most abundant division of bacteria was *Proteobacteria*, and the phylogenic positions of the 32 OTUs in *Proteobacteria* falling into four subdivisions of *Betaproteobacteria* (4), *Gammaproteobacteria* (12), *Deltaproteobacteria* (11), and *Epsilonproteobacteria* (5) are given in Fig. 1.

The most abundant group in *Proteobacteria* was *Gammaproteobacteria*, representing about 37.5% of the total *Proteobacterial* OTUs (Fig. 1B). Three of the *Gammaproteobacteria* OTUs (YSSB24, YSSB13, YSSB6) fell into the genus of *Pseudomonas,* which is widely distributed in various environments and plays an important role in nitrogen and heavy-metal recycling, including *Pseudomonas* sp. 2N1-1 (AJ419674) found in Antarctic marine sediment, GC06 (AY690672) from the salt marshes of Korea, DQ264567 found in subsurface groundwater during polylactate-stimulated chromate bioremediation, and AJ295645 found in uranium mill tailing [28]. In addition, bacteria was found related to YSSB13 (98% similarity), commonly observed in soil, fresh or marine water, and even hot-spring water. Similarly, three OTUs (YSSB30, YSSB8, YSSB70) were grouped into clades containing marine or intertidal sediment clones [20, 24, 34]. The highest similarity value with *Gammaproteobacteria* was found between clone YSSB24 and *Pseudomonas* sp. 2N1-1 collected from Antarctic marine sediment, with a 99% similarity.

*Deltaproteobacteria* (Fig. 1C), a predominant group in various marine sediments, was the second most abundant group of *Proteobacteria* (34.4% of total proteobacterial OTUs). Most of the clones were associated with sulfate-reducing bacteria (SRB) that play an important role in the mineralization of organic matter and degradation of pollutants in salt marshes and intertidal ecosystems. YSSB19 was grouped into a clade (98–99% similarity) containing many species of SRB obtained from salt marshes or intertidal sediments, except for clone QLS40-B36 that was found in saline sediment from Qinghai Lake in China. For YSSB19, the closest relative was *Desulfonema magnum* with a similarity of 91%. Three OTUs (YSSB1, YSSB15, YSSB16) formed a clade and were distantly related to *Desulfobulbaceae* (closest bacterium was YSSB1 with 97% similarity) previously found in intertidal sediment from Guanghwa Island in Korea and Wadden Sea in Germany [18], and one in Antarctic sediment [23]. Another OTU YSSB28 was clustered into a clade containing *Desulfuromonas chloroethenica* (92% similarity) and clone gubh2-AG-114 (AJ519664, 96% similarity) that were found in uranium mill tailings. Moreover, two OTUs (YSSB34, YSSB36) in a clade maintaining diverse bacteria observed in salt marshes, estuarine mud, inactive deep-sea hydrothermal vent chimneys, terrestrial volcano mud, and Yellowstone hot springs [8] showed no obvious association with any known bacteria in the subdivision of *Deltaproteobacteria*. In addition, OTU YSSB59 was grouped in a clade that was closely related to the *Nitrospina* family, where the closest clone (AB015243) was found in the deepest cold-seep marine sediment from the Japan Trench, with a similarity of 97% [13]. Three OTUs (YSSB18, YSSB38, YSSB48) also represented a deep branch supported by a high bootstrap value near the root of *Deltaproteobacteria*.

The *Betaproteobacteria*, representing 12.5% of the total proteobacterial OTUs, are shown in Fig. 1A. Two OTUs (YSSB67, YSSB69) were in a clade containing bacteria that are widely distributed in salt marshes, estuarine sediment, marine water, and sediment throughout the world [21, 22]. Clone YSSB16 was closely related to sulfur-oxidizing bacteria (SOB) that inhabit soil, fresh water, and harbor sediment, with a similarity ranging from 97 to 99% [30]. The close relatives of YSSB41 were plw-2 (DQ066963) (99% similarity) found in freshwater sediment from Lake Washington, and bacteria in wastewater involved in a nitrifying-denitrifying cycle (>96% similarity) [10].

Five OTUs belonged to *Epsilonproteobacteria* (Fig. 1D), and three of them (YSSB10, YSSB12, YSSB54) were found to be members of *Campylobacteraceae* collected from intertidal and hot deep-sea environments, such as a hydrothermal vent [2]. Moreover, OTU YSSB10 seemed intertide-specific, whereas YSSB3 seemed to be common in petroleum or trichloroethene-contaminated soil or water [33].

***Bacteroidetes***

The second most abundant group of bacteria was *Bacteroidetes*, accounting for 17.8% of the total OTUs in this study (Fig. 2A). Among them, seven OTUs (YSSB46, YSSB51, YSSB52, YSSB68, YSSB50, YSSB39, YSSB11) were *Flavobacteriaceae*, four (ASSB29, YSSB14, YSSB71,

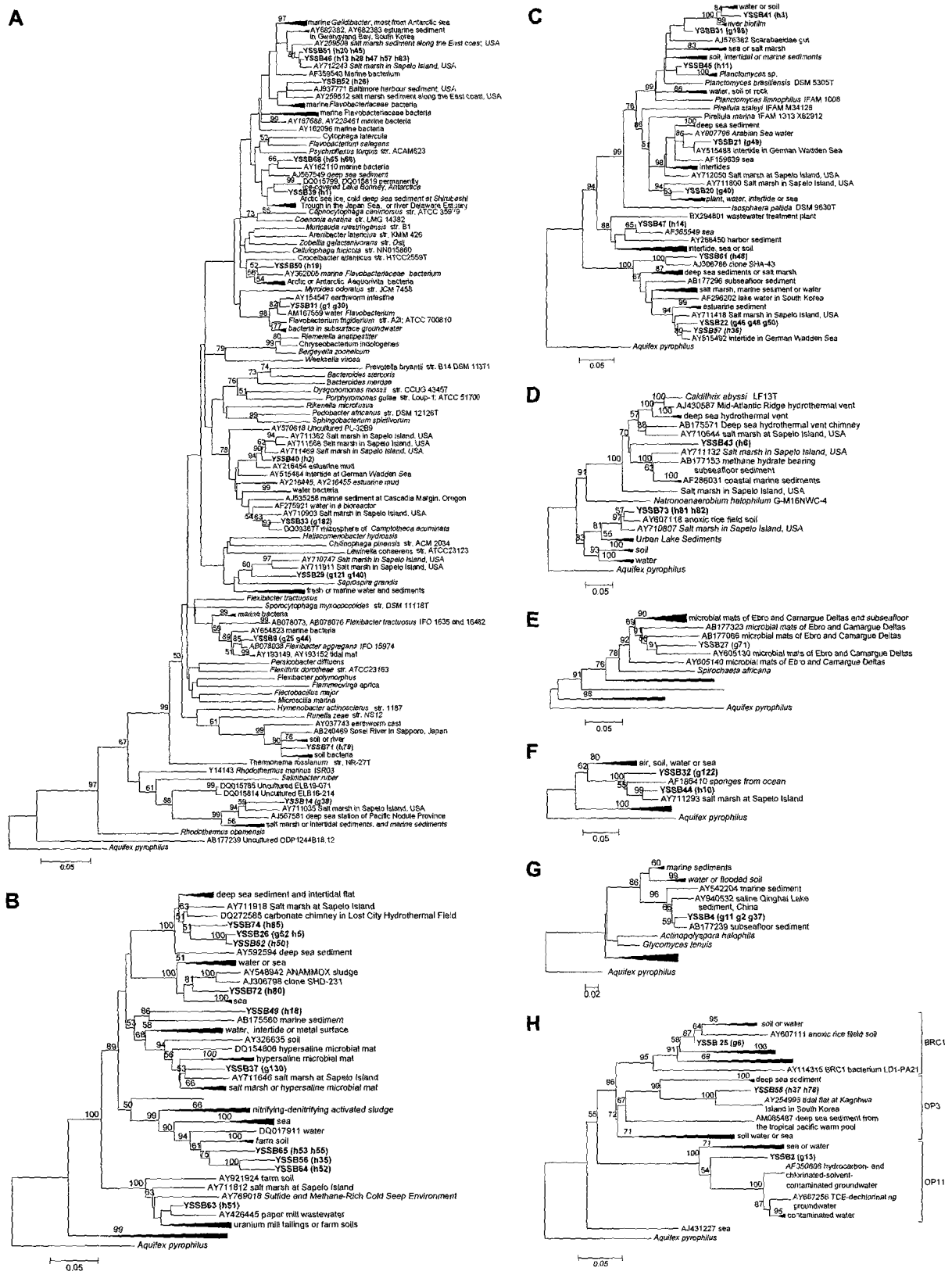


**Fig. 2.** Phylogenetic trees of *Bacteroidetes* (**A**), *Chloroflexi* (**B**), *Planctomycetes* (**C**), *Firmicutes* (**D**), *Spirochaetes* (**E**), *Acidobacteria* (**F**), *Actinobacteria* (**G**), and candidate divisions BRC1, OP11, OP3 (**H**).
Bars indicate nucleotide changes per 16S rDNA position.

YSB9) were *Sphingobacteriales*, and two could not be identified with any known group of *Bacteroidetes*. Among the *Flavobacteriaceae*, two OTUs (YSSB46, YSSB51) were clustered into a probably intertide-related clade containing an uncultured *Flavobacteria* clone, SIMO-706 (AY712243, 96% similarity), recovered from surface water at Sapelo Island and Dean Creek Marsh sediment along the east coast of the U.S. OTU YSSB52, was distantly related to *Gelidibacter* bacteria (87–89% similarity) that have been observed in many environments, such as salt marsh sediment along the east coast of the U.S. (AY259512), estuarine sediment from Gwangyang Bay in South Korea (AY682382, AY682383), harbor sediment from Maryland, U.S. (AJ937771), as well as in marine water (AF359540) [7, 21]. Another two OTUs (YSSB68, YSSB50) were clearly related to marine bacteria, although the similarity was less than 92%. However, OTU YSSB39 was a very interesting *Flavobacteriaceae* bacterium, with the highest similarity of 92–93% to bacteria residing in cold environments, such as ice-covered lakes or seas in the Arctic and Antarctic circle, even in deep-cold marine sediments. Only one OTU, YSSB11, belonged to bacteria found in fresh water environments (95–96% similarity).

Four OTUs (YSSB9, YSSB14, YSSB71, YSSB29) were *Sphingobacteriales*. YSSB9 belonged to the genus *Flexibacter* that is widely distributed in marine or tidal environments, YSSB14 and YSSB71 were closely related to bacteria found in marine sediment, and YSSB29 was found to be related to clones SIMO-374 and SIMO-1307 recovered from the salt marshes of Sapelo Island with a 60% bootstrap support. However, the similarity between YSSB29 and SIMO-374 or SIMO-1307 was much lower (83-84%) than the similarity between SIMO-374 and SIMO-1307 (91%), implying that a large group of ASSB29 bacteria may exist in intertidal environments.

Moreover, two OTUs (YSSB40 and YSSB33) showed no clear association with any known bacteria, even at a class level, although their subclades were found to be related to bacteria inhabiting intertidal and water or salt marsh environments, respectively.

### *Chloroflexi*

The third most abundant group of bacteria was *Chloroflexi* (13.7% of the total OTUs) (Fig. 2B). A total of ten OTUs of 12 clones were grouped into 5 clades. A phylogenic analysis of these clades showed that none of the sequences recovered in this study were intertide-related, except for YSSB37, distantly related to clone SIMO-2280 obtained from Sapelo Island, that formed an intertide-related clade with a 53% bootstrap value. Members of the OUT YSSB72 are widely distributed in soil, fresh or marine water, and sediments and probably act on dechlorination [27]. OTU YSSB49 had few relatives, and the closest clone IBCC-22 (85% similarity) was recovered from marine sediment from the hydrothermal conduit, Mid-Okinawa Trough of Japan [19].

### *Planctomycetes*

Nine OTUs from 11 clones were found to be the fourth most abundant bacteria within *Planctomycetes* (12.3% of the total OTUs) (Fig. 2C). The bacteria in *Planctomycetes* seem widely distributed in multiple environments and do not tend to form environment-specific groups, such as YSSB41 closely related to the water clone A24 recovered from nitrifying-denitrifying activated sludge (96% similarity) [10], or YSSB61 close to clone SHA-43 (92% similarity) acting on the dechlorination of 1,3-dichloropropane. Only one clade, containing YSSB22 and YSSB57, recovered in this study exhibited some type of intertide-related feature.

### Bacteria in Other Phyla

In addition to *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Planctomycetes*, 13% of the total OTUs belonged to seven divisions of bacteria: *Acidobacteria*, *Firmicutes*, *Actinobacteria*, *Spirochaetes*, and uncultured candidate divisions BRC1, OP3, and OP11.

Two OTUs (YSSB73, YSSB43) were found within *Firmicutes* (Fig. 2D), where the first was closely related to clones X3Ba18 and SIMO-1367 (95% similarity) recovered from an anoxic rice field and Sapelo Island, respectively, whereas the second showed no clear association to any known sequences in this phylum (<81% similarity).

One OUT, YSSB27, in *Spirochaetes* (Fig. 2E) was proximal to bacteria obtained from the Ebro and Camargue Delta microbial mats, with the highest similarity of 87%.

Two OTUs (YSSB32, YSSB44) were found within *Acidobacteria* (Fig. 2F), where the second may specifically inhabit an intertidal environment.

One OTU, YSSB4, belonging to *Actinobacteria* (Fig. 2G), was closely related to bacteria recovered from the subseafloor near the Peru margin (95% similarity) and saline Qinghai Lake sediment from China (92% similarity).

The remaining three OTUs (YSSB25, YSSB2, YSSB58) were within candidate phyla BRC1, OP11, and OP3, respectively (Fig. 2H). YSSB25 in BRC1 was closely related to clones (91–94% similarity) commonly recovered from flooded anoxic rice field soil [15]. YSSB2 within OP11 may relate to bacteria acting on dechlorination [14, 17]. YSSB58 in OP3 had a high similarity (91%) to clone BS1-0-34 recovered from tidal flats at Guanghwa Island [11].

## DISCUSSION

A phylogenetic analysis of the sequences recovered in this study and ten of their closest sequences disclosed some striking characteristics of the bacterial diversity in intertidal sediments:

(1) Intertidal sediments may contain special groups of bacteria. In other words, intertide-related bacteria may exist in an intertidal environment. Many clones constructed in this study were found to be closely related to clones recovered from the tidal flats at Guanghwa Island, Korea, salt marshes at Sapelo Island, U.S.A., and other intertidal environments. Twenty out of the total 73 OTUs in this study formed various intertide-related groups, such as YSSB46 and YSSB51 within *Bacteroidetes*.

(2) The intertidal bacteria were most likely derived from marine bacteria, even though the samples were collected from sediment. Except for 18%, the OTUs in this study formed separate phylogenic branches containing clones recovered from soil or fresh water, where 7% were widely distributed within sea, water, soil, and other environments, and 75% were associated with marine bacteria recovered from marine water or sediment, even marine hosts. Similar results were also found based on a partial phylogenetic analysis of other intertidal sequence libraries retrieved from the GenBank (data not shown) [11].

(3) The bacterial diversity in the test intertidal sediment was remarkably high. According to the results obtained in this study, a low Shannon index and diverse lineages found in the phylogenetic tree analysis, it was clear that the bacterial diversity in intertidal sediment is much higher than that in most other environments. However, the dominant groups of bacteria in the intertidal area seemed similar, including *Bacteroidetes* [11, 16], *Gammaproteobacteria*, and *Deltaproteobacteria* [6, 11, 12], which were demonstrated to be the major bacterial components in an intertidal environment.

In spite of these striking common characteristics, distinct patterns of bacterial diversity were found for different intertidal areas. For instance, the bacterial diversity in this study was significantly different from that for Sapelo Island and Guanghwa Island when using the LIBSHUFF program, where the diversity for the latter two was also distinct. Moreover, the structures and numbers of dominant bacteria were not completely consistent among the different intertidal areas. Some bacteria were particularly abundant in one area, yet not in another. Table 1 lists the phylogenetic differences between the bacterial 16S DNA sequences recovered from this study and those retrieved from the GenBank [11], which revealed a consanguineous relationship between an intertidal area and its surrounding environment. In addition, certain bacteria identified in the present study were found to be obviously involved in the degradation of organic matter and heavy metals, indicating that the intertidal sediment from the coast of the Yellow Sea, China was probably contaminated.

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