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**Full Paper**

# Bacterial populations in epilithic biofilms along two oligotrophic rivers in the Tohoku region in Japan

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**Bacterial populations in epilithic biofilms collected from two distinct oligotrophic rivers of Japan were studied using denaturing gradient gel electrophoresis (DGGE). PCR-DGGE of the 16S rRNA gene and subsequent sequencing analysis suggested that in freshwater biofilms, members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group were the most dominant, followed by those of α-, β-, γ-, and δ-Proteobacteria; Leptospiraceae; and unidentified bacteria. Members of the CFB group, α-Proteobacteria, and cyanobacteria/plastid DNA were also detected from the biofilms collected from the estuary site, but the species in these samples differed from those detected in biofilms in the freshwater areas of the rivers. A comparison between the determined sequences revealed that similar bacterial species existed in biofilms at different sites of a river, and identical species existed in biofilms of distinct rivers. The results suggested that bacterial species in biofilms found in the estuary were different from those found in the freshwater areas of the rivers; however, the common bacterial species were distributed in biofilms collected from not only different sites along the same river but also sites in distinct oligotrophic rivers.**

**Key Words**——bacterial communities; biofilm; estuary; river; 16S rRNA

## Introduction

Rivers are essential for human society since they provide water for consumption, agricultural, industrial and leisure activities, and carry wastewater away. Recent studies have revealed that various microbial communities exist in aquatic ecosystems, and they play important roles in the nutrient cycle, such as primary production, nitrification, and mineralization of organic matters (Meyer, 1994; Simon et al., 2002). Therefore, knowledge about the activities and distribution of microorganisms in rivers will enhance our understanding of entire river ecosystems and is beneficial for the maintenance of a healthy aquatic environment.

In aquatic systems, individual bacterial cells occur as both planktonic and attached cells. Biofilms formed on stones or on artificial substrates in rivers are composed of autotrophic and heterotrophic microorganisms in an extracellular polysaccharide matrix (Lock et al., 1984). In general, bacterial populations in biofilms have higher physiological activity and are more abundant than free-living bacterial cells (Araya et al., 2003; Costerton et al., 1987; Manz et al., 1993). Araya et al. (2003) presumed that surface-associated bacterial populations play a critical role in their study areas. It is

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also known that bacterial populations in freshwater biofilms are phylogenetically diverse (Brümmer et al., 2000; Manz et al., 1999).

A thorough understanding of the bacterial community in biofilms is expected to be useful for the development of indicators of health and safety of the river ecosystem. While substantial information is available on the compositions of bacterial communities in streams, rivers, and estuaries (Brümmer et al., 2000; Cottrell et al., 2005; Jones et al., 2007; Sekiguchi et al., 2002), most studies have focused on only 1 ecosystem each. Few reports have examined the bacterial populations in biofilms in the ecosystem in the entire length of rivers, from headwater to estuary. A description of the characteristics of biofilms in the ecosystems of the entire river will provide valuable information for future water management.

In recent years, culture-independent molecular techniques have been widely applied to ecological studies of microbial communities (Amann et al., 1995; Hugenholtz et al., 1998). Denaturing gradient gel electrophoresis (DGGE) and polymerase chain reaction (PCR) are useful for the identification of the dominant sequence types in the DNA extracted from environmental samples (Muyzer et al., 1993). PCR-DGGE also enables the rapid and simultaneous comparison of bacterial communities from different samples. In addition, PCR-DGGE along with sequencing analysis can be used to identify the dominant species in a biofilm community by comparing experimentally determined sequences with those registered on databases. This technique is a powerful tool, and has been developed and applied to study the diversity of bacteria in epilithic biofilms (Lyautey et al., 2005).

The objective of the present study is to determine the compositions of the bacterial communities in epilithic biofilms collected from the upstream to downstream areas of two oligotrophic natural rivers in Japan. Both rivers are located in the Tohoku region; however, one river runs through an urban area, while the other mainly runs through a rural area. The other objective is the comparison of the characteristics of the bacterial communities in the river running through the urban area and those through the rural area. For this, we used PCR-DGGE to target the 16S rRNA gene, and then performed sequencing analysis.

## Materials and Methods

*Study sites and sampling procedures*. The study was performed along the following two rivers: the Hirose River and the Kesen River. The two rivers do not cross.

The Hirose River is a tributary river in the Natori River system located in Miyagi Prefecture in the Tohoku region in Japan and is 45 km long. This river runs through Sendai City—the biggest city in the Tohoku region with a population of 1 million. A total of 5 sites (H1–H5) were studied upstream and downstream of Sendai City (Fig. 1A) on November 18, 2004. The center of Sendai City is located between H4 and H5. All the sites (H1–H5) were freshwater areas.

The Kesen River is located in Iwate Prefecture in the Tohoku region in Japan and is 47 km long. This river runs through Sumita Town and Rikuzentakata City with a population of about 30,000. This river is a clear stream and a popular fishing spot. Biofilm samples were collected at 4 sites (K1 and K3–K5) from the up-

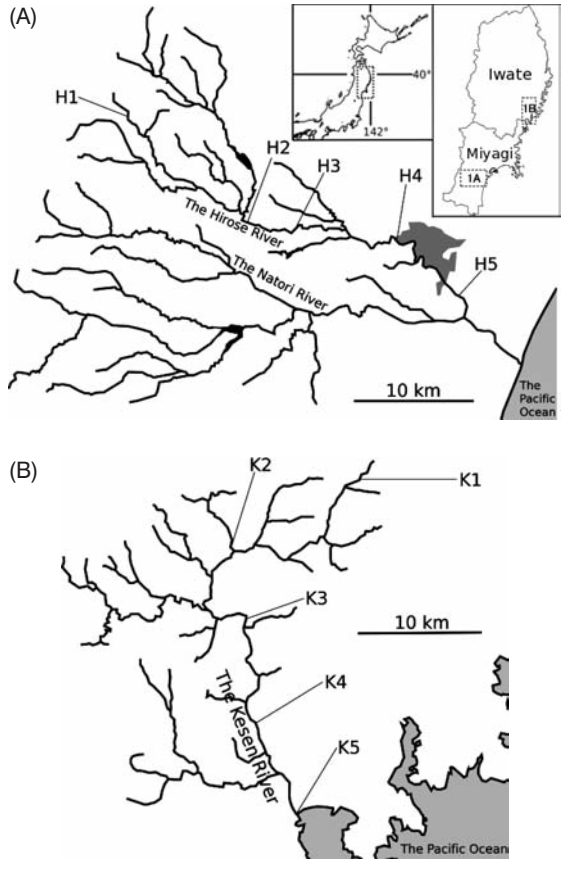


Fig. 1. Map of the Hirose River (A) and the Kesen River (B). The location of the sampling sites are shown. The center of Sendai City is shown as a gray region in Fig. A.

stream to downstream areas along the Kesen River and another site (K2) from the tributary (Fig. 1B) on December 11, 2004. Sites K1–K4 were freshwater areas, while site K5 was an estuary and contained brackish water.

Natural epilithic biofilms were collected from the above sites (water depth: 30–80 cm) by using sterile cutter blades and transferred to clean 15-ml tubes filled with river water at the same point. From each site, 1 or 2 biofilm samples were analyzed. When 2 samples were collected, 2 different stones were used; however, at site H5, the two samples were collected from the top and side of a single stone. Simultaneously, the water temperature at each site was measured with a Thermo Recorder (T&D Corporation, Japan), and 1.5-L water samples were also collected in order to assess water quality. The biofilm and water samples were transferred at 4°C and stored until analysis.

*Chemical analysis*. Water samples were used to analyze soluble biochemical oxygen demand (BOD) according to the protocol described by Japan Sewage Works Association (JSW, 1997), and to measure the concentrations of ammonium ($N\text{-}NH_4^+$), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), and total nitrogen (T-N) by using Hach reagents (Loveland, USA).

*DNA extraction*. The biofilm samples were disrupted by performing sonication 3 times at 4°C for 3 min with Bioruptor UCW-201 (Cosmo Bio). The disrupted samples were then centrifuged at 500 × *g* for 3 min, and 1.5 ml supernatant was transferred to 1.5-ml tubes. The tube was centrifuged at 10,000 × *g* for 5 min, and microbes were collected as pellets. After 5 rounds of freezing and thawing, DNA was extracted from the pellets with a MagExtractor™ -Genome- (TOYOBO) according to the manufacturer's instructions. The extracted DNA was dissolved in 50 μl sterile Milli-Q water and used as a PCR template.

*PCR*, *DGGE*, *and sequencing analysis*. The variable V3 region of the 16S rRNA gene was amplified using the primers 341F, which contains a GC clamp (5′-C GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG CCT ACG GGA GGC AGC AG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′). This primer set was designed to be specific for most bacteria, and a GC clamp was attached to the 5′ end of the forward primer for DGGE as described by Muyzer et al. (1993). A 50-μl volume of amplification reaction mixture contained 1 μl extracted DNA as a PCR template, 5 μl 10 × PCR buffer, each primer solution at a concentration of 0.25 μM, 0.2 mM of each dNTP, and 1.25 U TaKaRa Ex *Taq*® DNA polymerase (TaKaRa Bio, Inc.). For amplification of the 16S rRNA gene, a touchdown PCR program (Don et al., 1991; Sekiguchi et al., 2002) was implemented as follows: after initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, the annealing temperature for 20 s, and 72°C for 30 s were performed. The reaction mixture was then maintained at 72°C for 7 min. During the reaction cycle, the annealing temperature was decreased by 1°C from 65°C to 56°C for every second cycle in the first 20 cycles. The annealing temperature was 55°C in the last 10 cycles. The PCR products were evaluated by electrophoresis in a 1.2% agarose gel, followed by staining with ethidium bromide solution and visualization using an ultraviolet (UV) transilluminator.

DGGE was performed using the D-code Universal Mutation Detection System (Bio-Rad Laboratories, Inc.). A polyacrylamide gel [8% (wt/vol) of a 37.5∶1 acrylamide-bisacrylamide mixture in 1× Tris-acetate-ethylene diamine tetraacetic acid (EDTA) (TAE) buffer (0.04 M Tris base, 0.02 M acetate, and 1.0 mM EDTA)] was used. Denaturant gradients from 30% to 70% [100% denaturant contained 7 M urea plus 40% (wt/vol) formamide] in the direction of electrophoresis were used for the analysis of the 16S rRNA gene. After the application of 30 μl PCR products, the gels were subjected to a constant voltage of 100 V for 8 h at 60°C. Following electrophoresis, the gels were stained for 10 min with ethidium bromide and photographed under UV illumination.

Visible bands were excised from the gel by using sterilized cutter blades, transferred to 50 μl sterile water, and incubated overnight at 4°C. The eluted DNA was reamplified using the protocol described above, and the purity of the PCR products was checked using DGGE. This procedure was repeated until a single band appeared. After this step, PCR was conducted with the primer set 341F-518R without the GC clamp. Subsequently, the amplicons were purified with MagExtractor™-PCR & Gel Clean up- (TOYOBO) and sequenced using BigDye® terminator cycle sequencing kit v1.1 (Applied Biosystems) according to the manufacturer's instructions on an ABI 310 Genetic Analyzer (Applied Biosystems). Sequence accuracy was confirmed using two-directional sequencing.

*Homology search and phylogenetic analysis*. All the sequences determined in this study were compared with similar sequences registered in the DDBJ/

EMBL/GenBank databases by using BLAST from the DNA Data Bank of Japan (DDBJ) web page (http://blast.ddbj.nig.ac.jp/top-j.html). By using the ClustalW alignment program, alignments were constructed for the *Cytophaga-Flavobacteria-Bacteroides* (CFB) group and other divisions of the Bacteria by using the determined sequences and homologous sequences to closely related bacteria. Phylogenetic analyses were conducted with MEGA4 (Tamura et al., 2007).

*Nucleotide sequence accession numbers*. The nucleotide sequences of 16S rRNA gene obtained in this study were registered in DDBJ/EMBL/GenBank databases under accession numbers AB458598 to AB458647.

## Results

*Environmental variables*

Physical and chemical characteristics of the Hirose and Kesen rivers were assessed (Table 1). The temperatures of both rivers were approximately constant from the upstream to downstream areas. The temperature of the Hirose River ranged from 9.4°C to 11.2°C, and that of Kesen River, from 7.2°C to 9.2°C. BOD in the Hirose River gradually increased from site H1 (0.38 mg/L) to H4 (1.56 mg/L), and decreased at site H5 (1.01 mg/L). In the case of the Kesen River, the BOD was 1.31 mg/L at site K1; it decreased at site K2 (1.19 mg/L), and thereafter, it progressively increased. $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$ concentrations were undetectable at all the study sites. The $N\text{-}NO_3^-$ and T-N concentrations were relatively higher in the Kesen River than in the Hirose River, but were nevertheless low. This shows that the water of the Hirose River was as clear as that of the Kesen River.

*PCR-DGGE analysis, sequencing and identification of DGGE fragments*

The partial fragments of 16S rRNA gene were amplified by using the universal primer set, and DGGE of the PCR products was performed. The DGGE profiles of the biofilm bacterial communities formed on stones along the Hirose and Kesen rivers are shown in Figs. 2 and 3, respectively. Several bands were obtained from each DNA extract. The DGGE profiles of the samples collected from all sites along the Hirose River (Fig. 2) were different. With regard to the DGGE profiles of the Kesen River (Fig. 3), the 2 samples collected at sites K3 and K4 exhibited similar band patterns.

DNA sequence determinations of all visible PCR bands were tried. A total of 47 bands (Hirose River: 17 bands and Kesen River: 30 bands), which are labeled with numbers in Figs. 2 and 3, were sequenced successfully. No sequence could be determined from lane H2. The sequences were named according to the sampling site and the label number of the band (e.g., the K2_1 sequence was determined from the band labeled "1" in lane K2). Two sequences each time were obtained from bands K1_4, K3_4, and K4_8; this was because they contained $>1$ DNA sequence due to the comigration of DNA from different bacterial species. The sequences were separated using multiple PCR-DGGE. In total, 50 sequences were determined from 47 bands, and a homology search was conducted (Table 2). The most frequently determined sequences were closely related to the 16S rRNA gene sequences of uncultured microorganisms obtained from environmental samples that were collected from sources such

Table 1. Physical and chemical characteristics of the study sites on the day of sampling.

| River | Site | Temp (°C) | BOD (mg/L) | $NH_4$-N (mg/L) | $NO_2$-N (mg/L) | $NO_3$-N (mg/L) | T-N (mg/L) |
|---|---|---|---|---|---|---|---|
| The Hirose River | H1 | 9.4 | 0.378 | N.D.[a] | N.D. | 0.1 | N.D. |
| | H2 | 10.9 | 1.14 | N.D. | N.D. | 0.2 | 0.1 |
| | H3 | 10.6 | 1.44 | N.D. | N.D. | 0.2 | 0.2 |
| | H4 | 11.2 | 1.56 | N.D. | N.D. | 0.2 | 0.4 |
| | H5 | 10.7 | 1.01 | N.D. | N.D. | 0.2 | 0.2 |
| The Kesen River | K1 | 8.6 | 1.31 | N.D. | N.D. | 0.2 | 0.2 |
| | K2 | 7.2 | 1.19 | N.D. | N.D. | 0.8 | 1.3 |
| | K3 | 7.5 | 1.21 | N.D. | N.D. | 0.6 | 0.8 |
| | K4 | 7.4 | 1.39 | N.D. | N.D. | 0.6 | 0.9 |
| | K5 | 9.2 | 1.42 | N.D. | N.D. | 0.3 | 0.5 |

[a]The values less than 0.05 were omitted and considered as "not detected."

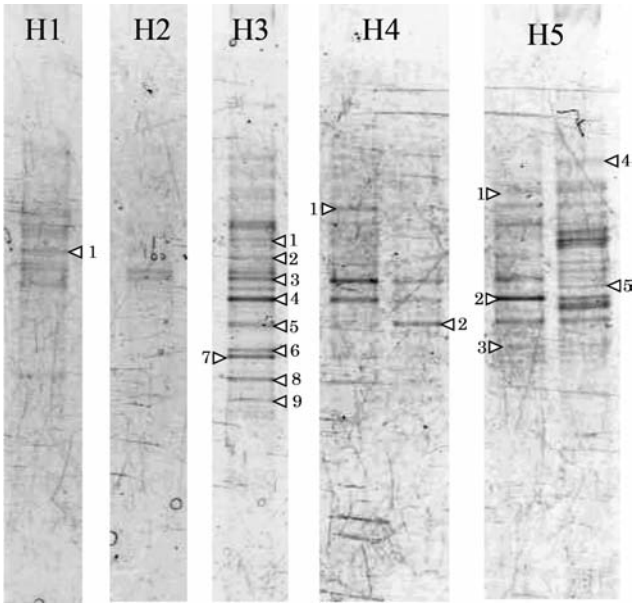


Fig. 2. DGGE-band profiles for samples obtained from the Hirose River.

The indicated bands were excised and sequenced.

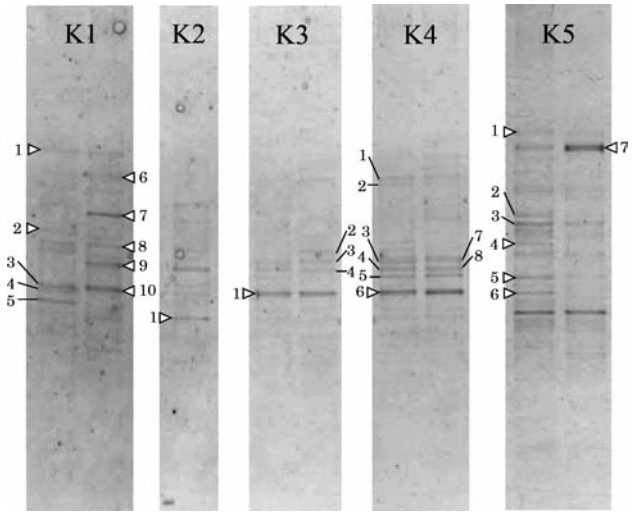


Fig. 3. DGGE-band profiles for samples obtained from the Kesen River.

The indicated bands were excised and sequenced.

as lakes (Eiler and Bertilsson, 2007; Eiler et al., 2006), rivers (Beier et al., 2008; Cottrell et al., 2005; O'Sullivan et al., 2002), freshwater sources (Aslam et al., 2005; Horner-Devine et al., 2003; Osaka et al., 2006), estuaries (Kisand and Wikner, 2003; Kisand et al., 2002), coasts (Musat et al., 2006), and soil (Futamata et al., 2003; Yergeau et al., 2007). In particular, the uncultured CFB group bacterium clone TAF-B64, whose sequence was related to K1_1, was also detected in the biofilms (O'Sullivan et al., 2002). Furthermore, the sequences obtained from samples collected at site K5 (brackish-water area) tended to show high similarity to those from the samples collected from areas such as estuaries and intertidal areas where seawater enters.

Some similar sequences were observed at different sites along the Kesen River. The following pairs of sequences were identical: (1) K3_1 and K4_6, (2) K3_4_1 and K4_8_2, and (3) K3_4_2 and K4_4. Additionally, the sequences K3_1 and K4_6 were not identical to K1_10 and K3_2, but were more than 98% similar. Furthermore, several identical sequences were obtained from both the Hirose and Kesen rivers, although these two rivers do not cross. The sequences H3_5, H5_2, and H5_5 amplified from biofilm samples collected from the Hirose River were identical to sequences K1_3, K4_5, and K4_3, respectively, which were amplified from biofilms collected from the Kesen River. This shows that closely related bacterial species that possessed these 16S rRNA sequences were dominant in the biofilm communities of both rivers.

Neighbor-joining trees for CFB group (Fig. 4) and for other bacterial divisions (Fig. 5) were constructed. Phylogenetic analysis revealed that the sequences determined in this study belonged to the following categories: CFB group (37 sequences), α-Proteobacteria (4 sequences), β-Proteobacteria (1 sequence), γ-Proteobacteria (1 sequence), δ-Proteobacteria (1 sequence), unidentified Proteobacteria (1 sequence), Leptospiraceae (2 sequences), cyanobacteria/algal plastid DNA (1 sequence), and unclassified bacteria (2 sequences). Many bacteria belonging to the CFB group and to the Leptospiraceae family were detected in biofilm samples collected from both rivers.

## Discussion

The examination of the differences in the bacterial communities within a single river and those in multiple rivers can offer insights into the general characteristics of biofilms. In this study, we examined the bacterial populations in biofilms from two rivers in Japan by using the molecular technique PCR-DGGE. One or two biofilm samples were collected from 2 different stones for the analysis in each sampling point. Biofilm samples collected in the same sampling point showed similar DGGE profiles. However, further study is required to clarify the influence of various factors such as stone materials to bacterial communities on the sur-

Table 2. Sequence similarities to the nearest relative and the phylogenetic affiliations of the DNA recovered from the DGGE gel.

| River | Site | No. | Similarity (%) | Nearest relative | Accession no. | Isolation source | Phylognetic affiliation[a] | References |
|---|---|---|---|---|---|---|---|---|
| The Hirose River | H1 | H1_1 | 96 | Uncultured bacterium clone N2-86 | EU443085 | Nam Co Lake water | β-Proteobacteria | Unpublished |
| | H3 | H3_1 | 98 | Uncultured soil bacterium clone S20 | AB080337 | TCE/phenol-degrading soil | CFB | Futamata et al., 2003 |
| | | H3_2 | 97 | Uncultured bacterium clone SRRB41 | AB240514 | Root-base of Phragmites at Sosei River | CFB | Unpublished |
| | | H3_3 | 100 | Uncultured Flavobacteria bacterium clone LiUU-22-42 | EF061041 | Productive lake | CFB | Eiler et al., 2006 |
| | | H3_4 | 98 | Uncultured Cytophagales ESR 19 | AF268303 | Lake Esrum | CFB | Riemann and Winding, 2001 |
| | | H3_5 | 100 | Uncultured Cytophagales ESR 20 | AF268304 | Lake Esrum | CFB | Riemann and Winding, 2001 |
| | | H3_6 | 100 | Uncultured Flavobacterium sp. clone GOBB3-CL208 | AF388900 | Estuarine dilution cultures | CFB | Kisand and Wikner, 2003 |
| | | H3_7 | 100 | Uncultured bacterium clone W-Btb7_09 | DQ017917 | Upland stream | CFB | Beiser et al., 2008 |
| | | H3_8 | 95 | Antarctic bacterium strain 3C8 | EU636038 | Collins glacier | γ-Proteobacteria | Unpublished |
| | | H3_9 | 100 | Uncultured bacterium clone cams11-1 | AY544212 | ?[c] | unclassified Bacteria | Unpublished |
| | H4 | H4_1 | 90 | "*Candidatus* Magnospira bakii" clone BM15 | AF087096 | ?[c] | CFB | Unpublished |
| | | H4_2 | 99 | Uncultured Flavobacterium sp. clone LXA | EF424249 | Rotten algae | CFB | An et al., 2008 |
| | H5 | H5_1 | 100 | Uncultured Bacteroidetes bacterium clone B76 | AY562309 | The Delaware River | CFB | Cottrell at al., 2005 |
| | | H5_2 | 100 | *Flavobacterium* sp. EP131 | AF493665 | United Kingdom: Wales, River Taff | CFB | Unpublished |
| | | H5_3 | 99 | Uncultured Flavobacterium sp. clone GOBB3-CL208 | AF388900 | Estuarine dilution cultures | CFB | Kisand and Wikner, 2003 |
| | | H5_4 | 99 | Uncultured Leptonema sp. clone BF.S74 | FM175101 | Rivulet | Leptospiraceae | Unpublished |
| | | H5_5 | 95 | *Flavobacterium* sp. BF.107 | FM173271 | Biofilm of core of Westerhoefer Bach | CFB | Unpublished |
| The Kesen River | K1 | K1_1 | 100 | Uncultured CFB group bacterium clone TAF-B64 | AY038769 | The river Taff | CFB | O'Sullivan et al., 2002 |
| | | K1_2 | 97 | Uncultured alpha proteobacterium clone MS046A1_E02 | EF701977 | Human gastrointestinal resection specimen | α-Proteobacteria | Frank et al., 2007 |
| | | K1_3 | 100 | Uncultured Cytophagales ESR 20 | AF268304 | Lake Esrum | CFB | Riemann and Winding, 2001 |
| | | K1_4_1[b] | 97 | Uncultured proteobacterium clone GASP-WA1W3_H08 | EF072440 | GASP Watkinsville sampling site | unclassified Proteobacteria | Unpublished |
| | | K1_4_2[b] | 96 | Uncultured Bacteroidetes bacterium clone AI-1M_H05 | EF219724 | Soil environments under mosses | CFB | Yergeau et al., 2007 |
| | | K1_5 | 100 | Uncultured Cytophagales ESR 21 | AF268305 | Lake Esrum | CFB | Riemann and Winding, 2001 |
| | | K1_6 | 96 | Uncultured freshwater bacterium clone 965005D05.×1 | DQ065453 | Freshwater | CFB | Horner-Devine et al., 2003 |
| | | K1_7 | 94 | Uncultured Sphingobacterium sp. clone DR546AS34-2 | DQ128143 | Ultradeep mine | CFB | Onstott et al., 2003 |

Table 2. Continued.

| River | Site | No. | Similarity (%) | Nearest relative | Accession no. | Isolation source | Phylognetic affiliation[a] | References |
|---|---|---|---|---|---|---|---|---|
| | | K1_8 | 98 | Uncultured bacterium clone 12C-M65 | AB205903 | Denitrifying activated sludge | CFB | Osaka et al., 2006 |
| | | K1_9 | 99 | *Bdellovibrio* sp. oral clone CA006 | AF385539 | Oral environment | δ-Proteobacteria | Paster et al., 2002 |
| | | K1_10 | 98 | Uncultured freshwater bacterium clone 965003D08.×1 | DQ065317 | Freshwater | CFB | Horner-Devine et al., 2003 |
| | K2 | K2_1 | 97 | Uncultured bacterium clone MA73_2004T8d_E02 | EF378554 | Soil | CFB | Unpublished |
| | K3 | K3_1 | 99 | Uncultured freshwater bacterium clone 965003D08.×1 | DQ065317 | Freshwater | CFB | Horner-Devine et al., 2003 |
| | | K3_2 | 98 | Uncultured freshwater bacterium clone 965003D08.×1 | DQ065317 | Freshwater | CFB | Horner-Devine at al., 2003 |
| | | K3_3 | 98 | Uncultured bacterium clone PO-19 | EU375400 | Lake | CFB | Unpublished |
| | | K3_4_1[b] | 97 | Uncultured freshwater bacterium clone 965002H11.×1 | DQ065295 | Freshwater | CFB | Horner-Devine et al., 2003 |
| | | K3_4_2[b] | 100 | *Blastomonas natatori* DSM 3183 type strain | Y13774 | Freshwater swimming pool | α-Proteobacteria | Yurkov et al., 1997 |
| | K4 | K4-1 | 100 | Uncultured Leptonema sp. clone BF.S74 | FM175101 | Rivulet | Leptospiraceae | Unpublished |
| | | K4-2 | 100 | *Flectobacillus* sp. HTCC553 | AY584584 | Crater Lake | CFB | Page et al., 2004 |
| | | K4-3 | 95 | *Flavobacterium* sp. BF.107 | FM173271 | Biofilm of core of Westerhoefer Bach | CFB | Unpublished |
| | | K4-4 | 100 | *Blastomonas natatori* DSM 3183 type strain | Y13774 | Freshwater swimming pool | α-Proteobacteria | Yurkov et al., 1997 |
| | | K4-5 | 100 | *Flavobacterium* sp. EP131 | AF493665 | River Taff | CFB | Unpublished |
| | | K4-6 | 99 | Uncultured freshwater bacterium clone 965003D08.×1 | DQ065317 | Freshwater | CFB | Horner-Devine et al., 2003 |
| | | K4_7 | 92 | Uncultured Bacteroidetes bacterium clone LiUU-14-62 | AY874003 | Freshwater bloom | unclassified Bacteria | Eiler et al., 2006 |
| | | K4_8_1[b] | 95 | Uncultured bacterium clone MA73_2004T8d_E02 | EF378554 | Soil sample | CFB | Unpublished |
| | | K4_8_2[b] | 97 | Uncultured freshwater bacterium clone 965002H11.×1 | DQ065295 | Freshwater | CFB | Horner-Devine et al., 2003 |
| | K5 | K5-1 | 92 | *Flexibacter litoralis* strain IFO 15988 | AB078056 | Seawater aquarium | CFB | Nakagawa et al., 2002 |
| | | K5-2 | 98 | *Flavobacterium granuli* strain Kw05 | AB180738 | Freshwater | CFB | Aslam et al., 2005 |
| | | K5-3 | 99 | *Erythrobacter longus* strain JCM 6170 | D12699 | Seaweed | α-Proteobacteria | Kawasaki et al., 1992 |
| | | K5-4 | 95 | Uncultured bacterium clone SS1_B_08_26 | EU050904 | Sediment from the Kings Bay | CFB | Unpublished |
| | | K5-5 | 98 | *Flavobacterium* sp. GOBB3-209 | AF321038 | Estuarine dilution cultures | CFB | Kisand et al., 2002 |
| | | K5-6 | 98 | Uncultured Cytophagales bacterium clone Sylt 1 | AM040097 | Intertidal sandy sediments | CFB | Musat et al., 2006 |
| | | K5-7 | 98 | Uncultured phototrophic eukaryote clone UA01; plastid | DQ269056 | Surface of marine macro-alga | Cyanobacteria/ Plastid DNA | Unpublished |

[a]Phylogenetic affiliation was determined by phylogenetic analysis (Fig. 2 and 3). [b]Because of comigration of DNA, 3 bands consisted of multiple sequences. [c]?, nearest relative could not be determined.

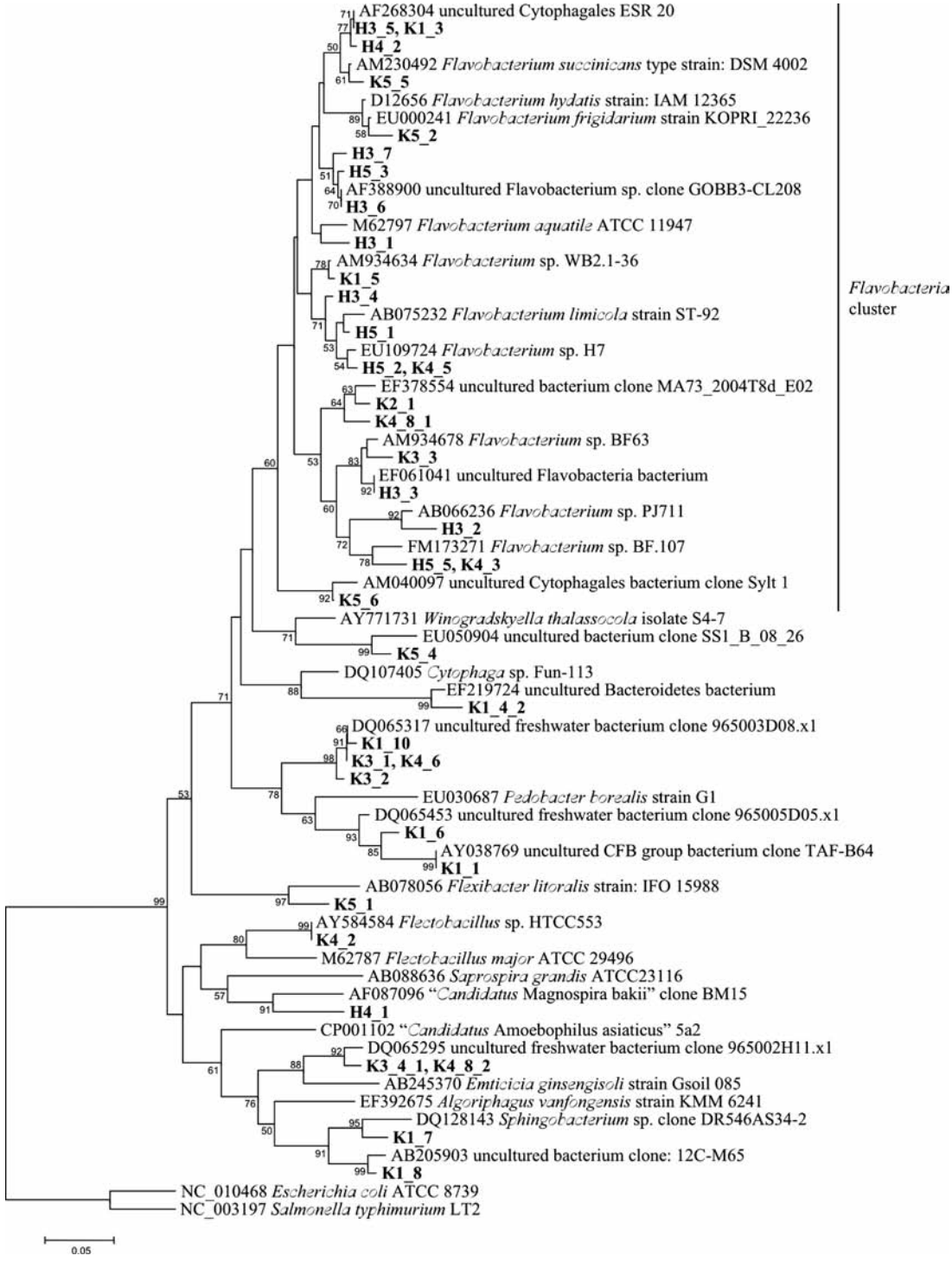


Fig. 4. Neighbor-joining tree illustrating the genetic relationships among members of the CFB group.

The tree was constructed using MEGA4 (Tamura et al., 2007) and was based on the partial 16S rRNA gene sequences determined. The sequences determined in this study are indicated by boldface and similar sequences from the databases are represented with their respective accession numbers. The tree is rooted with the 16S rRNA gene sequences of *Escherichia coli* and *Salmonella typhimurium*. Bootstrap values are based on 2,000 replicates; values greater than 50 are shown.

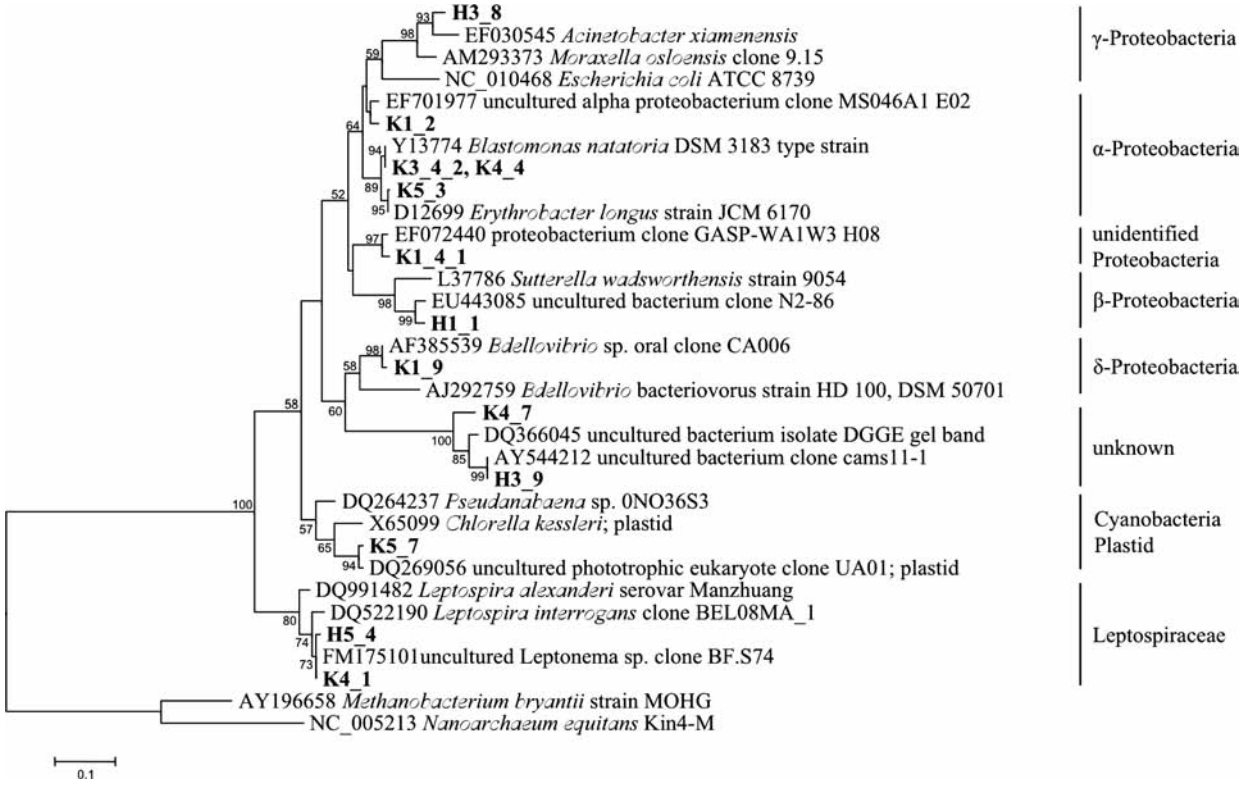


Fig. 5. Neighbor-joining tree illustrating the genetic relationships among bacterial species, except for those of the CFB group.

The tree was prepared according to the method given in Fig. 4. The tree is rooted with the archaeal 16S rRNA gene sequences of *Methanobacterium bryantii* and *Nanoarchaeum equitans*. The determined sequences are indicated by boldface and similar sequences from the database are represented with their respective accession numbers.

face of stones.

It is known that PCR-based techniques can create biases in steps such as nucleic acid extraction and PCR amplification (Ishii and Fukui, 2001; Martin-Laurent et al., 2001). Furthermore, the existence of multiple heterogeneous copies of the 16S rRNA gene within a genome is also likely to introduce biases (Case et al., 2007). Lyautey et al. (2005) observed that the amounts of DNA and PCR templates used during DGGE have to be standardized in order to obtain reproducible and consistent DGGE profiles. We did not consider such parameters in this study, and therefore, it was difficult to differentiate bacterial community structures among the sites by comparing the DGGE-band patterns. However, we obtained valuable information regarding the bacterial members present in the biofilm community by using DGGE-band sequencing and subsequent phylogenetic analysis.

In this study, members of the CFB group were most frequently detected (32 bands) in freshwater biofilms collected from both of the rivers; followed by members of α-Proteobacteria (3 bands) in the Kesen River, Leptospiraceae (2 bands) in both rivers, and other categories (β-, γ-, δ-Proteobacteria, and unclassified bacteria). Compared with members of the other categories, members of the CFB group were observed more abundantly in biofilms from freshwater samples and may be regarded as the dominant species in the studied biofilms. α-Proteobacteria was prevalent in the Kesen River; these bacteria were detected from freshwater regions (sites K1, K3, and K4) throughout the river. Surprisingly, only 1 sequence (H1_1) belonged to the group of β-Proteobacteria; these bacteria are usually abundant in freshwater biofilms all over the world (Araya et al., 2003; Brümmer et al., 2000; Gao et al., 2005; Manz et al., 1999).

Our result is consistent with the results reported by O'Sullivan et al. (2002). They investigated the bacterial diversity of River Taff epilithon and observed that the CFB bacteria were the most abundant, representing 25% of their constructed clone library; the second-most abundant bacteria were those belonging to

α-Proteobacteria. It is also reported that bacteria belonging to the CFB group and α-Proteobacteria are dominant in mature freshwater biofilms (Manz et al., 1999). Bacteria belonging to the CFB group in biofilms exhibit seasonal patterns, in addition high densities of these bacteria are frequently observed in winter (Brümmer et al., 2000; Rubin and Leff, 2007). Members of the CFB group are well adapted to both cold and oligotrophic ecosystems, and they are not only abundant in winter but are also found in high numbers in glacier ice (Battin et al., 2001). Similarly, it is also reported that α-Proteobacteria were more abundant in low-nutrient or cold environments (Gao et al., 2005; Rotthauwe et al., 1997). These conditions probably facilitate the growth of the bacteria belonging to the CFB group and α-Proteobacteria. Thus, the results that the members belonging to the CFB group were most frequently observed in both rivers and that α-Proteobacteria were prevalent in the Kesen River may be attributed to the conditions during sampling.

In the case of the brackish-water region (site K5), 5 of the 7 sequenced bands corresponded to the CFB group; of the remaining 2, 1 band corresponded to α-Proteobacteria and the other, to cyanobacteria/plastid DNA. Bacteria belonging to the CFB group are often abundant in particle-attached bacterial communities in marine and estuary ecosystems (Crump et al., 1999; Rath et al., 1998). Jones et al. (2007) observed that the members of α-Proteobacteria were most abundant in biofilms formed on artificial surfaces in an estuary. Although members of the CFB group and α-Proteobacteria were identified from site K5 as well as from freshwater sites, they tended to resemble microorganisms collected from areas such as estuaries and intertidal areas (Table 2). It was suggested that the members of biofilm communities in freshwater and brackish water differ at the species level even if they are similar at the class or family level.

Bacteria belonging to the CFB group are known to be chemoorganotrophic and especially proficient in degrading a variety of complex organic substrates such as cellulose, chitin, and pectin (Kirchman, 2002). In our study, the members of the CFB group were suggested as one of the most abundant species in biofilms from both freshwater and brackish-water areas; therefore, this bacterial group may be primarily responsible for heterotrophic activity and especially play an important role in the turnover of organic matter. In the case of α-Proteobacteria, DNA sequences closely related to those of *Blastomonas natatoria* were observed in sites K3 and K4, which were located in the central parts of the Kesen River (Table 2). *B. natatoria* strains isolated from freshwater biofilms are involved in coaggregation between other groups of bacteria (Rickard et al., 2002). The ability to coaggregate may function in the integration and establishment of bacteria in biofilms during the developmental stages (Rickard et al., 2003). The observed bacterial species that were related to *B. natatoria* might contribute to biofilm formation in the freshwater areas of the Kesen River.

Phylogenetic analysis revealed that the compositions of the bacterial communities in biofilms from both rivers were very diverse. Some sequences of the biofilm samples from different study sites along the Kesen River (Figs. 4 and 5) were identical (K3_1 and K4_6, K3_4_1 and K4_8_2, and K3_4_2 and K4_4). In addition, K1_10, K3_1, K4_6 and K3_2 formed a cluster (Fig. 4) and showed $>$98% similarity. These results suggested that similar bacterial species developed in biofilms from different freshwater sites in the Kesen River. Interestingly, several identical sequences (H3_5 and K1_3, H5_2 and K4_5, and H5_5 and K4_3) were also identified from the Kesen and Hirose rivers, even though these rivers do not cross (Figs. 4 and 5). Gao et al. (2005) observed that the benthic structure was extremely similar among different sites in a given stream, while large differences were observed in the bacterial composition in different streams. They also observed that some bands at similar locations on the DGGE gel were found in samples collected from multiple streams. Zwart et al. (2002) compared the 16S rRNA gene sequences obtained from planktonic bacteria of several freshwater ecosystems with the sequences in global databases, and observed that common planktonic bacteria are distributed in diverse freshwater environments around the world. In addition to the observation of common bacterial species in the two different rivers, we also discovered that the sequence K1_1 was identical to that of an uncultured bacterium clone TAF-B64 belonging to the CFB group, which was detected from a biofilm in the River Taff in the United Kingdom (O'Sullivan et al., 2002). Our data suggested that the common bacterial species might be distributed in biofilms of different habitats, as is the planktonic bacterial community. Similar environmental factors in the Hirose and Kesen rivers along with a low-nutrient environment (Table 1) might explain the similar dominant organisms in biofilms. On the other hand,

some sequences were detected at a specific site; for example K1_7 and K1_8 were only detected at upstream site K1. The microbial community in biofilm might reflect environmental characteristics that were not analyzed in this study such as turbidity, dissolved oxygen and phosphate. The acquisition of more detailed sequences and water quality data will enable the determination of the similarities and differences between the bacterial species in the biofilms in diverse river ecosystems in the world.

The 2 sequences which were phylogenetically similar to the family Leptospiraceae were detected from the two rivers. This family consists of both saprophytic and pathogenic species that affect animals and humans (Levett, 2001). The pathogenic species cause leptospirosis, a widespread zoonosis, which is a major concern in the developing countries in South-East Asia and South America. Leptospiral transmission occurs through direct contact with animal reservoirs (Levett, 2001) or exposure to urine-contaminated water (Trevejo et al., 1998). Leptospirosis is associated with rivers, and outbreaks of the infection have been reported among athletes practicing water sports and people performing a variety of recreational activities (Morgan et al., 2002; Yanagihara et al., 2007). Leptospires can survive for long periods of time in freshwater environments (Trueba et al., 2004). Ristow et al. (2008) reported that leptospires can form biofilms, and this ability might help pathogenic strains to survive in environments outside the host. We obtained only partial sequences of the 16S rRNA gene; therefore, it could not be determined whether the species detected in this study were pathogenic. However, since environmental biofilms may act as temporary reservoirs for pathogens such as pathogenic leptospires, research should be conducted from an ecological as well as a hygienic viewpoint to evaluate the safety of river biofilms.

In conclusion, by using PCR-DGGE targeting the eubacterial 16S rRNA gene, we observed that bacteria belonging to the CFB group were the most frequently detected in freshwater and estuary biofilms, and that $\alpha$-Proteobacteria were prevalent in the Kesen River. DNA-sequencing analysis revealed that the dominant bacterial species in the estuary site was different from the dominant species in freshwater areas. A comparison of the determined sequences suggested that similar bacterial species existed in biofilms at different sites of a river, and that identical species existed in distinct rivers. Further bacterial study that takes into account the environments surrounding river biofilms will provide valuable information for describing the general characteristics of biofilms, and this information will be useful for maintaining and managing the health and safety of river ecosystems.

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