Comparative Analysis of Bacterial Diversity in Freshwater Sediment of a Shallow Eutrophic Lake by Molecular and Improved Cultivation-Based Techniques†

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Comparative analysis of bacterial diversity in freshwater sediment collected from a shallow eutrophic lake was performed by using 16S rRNA gene clone library and improved cultivation-based techniques. Our study demonstrated that the use of gellan gum as a gelling reagent instead of agar was more effective at increasing culturability, cultivating a diverse array of novel microbes, and reducing the gaps of the results between molecular and cultivation-based analyses.

Prokaryotes are among the most important contributors to the transformation of complex organic compounds and minerals in freshwater sediments (22, 34). Therefore, investigating microbial structure and function in freshwater sediments is of great importance for gaining a better general understanding of aquatic ecosystems.

Although efforts have been made to reveal the microbial ecosystems in freshwater sediments on the basis of traditional cultivation methods (21, 45), it is now widely recognized that only 0.001 to 15% (0.25% in sediments) of the total cell counts in environmental samples can be cultured (3). Hence, the traditional cultivation methods cannot be directly applied to whole microbial diversity analyses. However, cultivation-based study remains important, since the ecological role of prokaryotes in natural environments can be estimated only when they are successfully cultivated and characterized.

The culture-independent molecular approaches based on small-subunit rRNA have also been used for studies of microbial ecology in freshwater sediments (1, 31, 38, 39, 45). However, information on microbial compositions in freshwater sediments is limited, since previous studies have mainly focused on some particular functional groups such as nitrifiers, denitrifiers, sulfate reducers, methanogens, and methanotrophs (8, 16, 29, 38, 45, 49). There are several studies on overall bacterial communities in freshwater sediments (33, 45, 50), but all of them are based solely on molecular analyses.

In this study, we performed comparative analyses of the bacterial diversity of freshwater sediment of a shallow eutrophic lake on the basis of 16S rRNA gene analysis and modified cultivation techniques that improved the culturability of hitherto unknown species present in the ecosystem. In particular,

we demonstrated the effectiveness of using gellan gum as a gelling reagent in a culture-dependent analysis of bacterial diversity in freshwater sediment.

Sampling site and sediment samples. Sediment samples were collected with an Eckman grabber from Lake Kasumigaura (36°08.07′N, 140°20.62′E), an important reservoir of water and a typical eutrophic freshwater lake in Japan, on 30 November 2002. Lake Kasumigaura is the second largest lake (lake area, 219.9 km^2; catchment area, 2,135 km^2) in Japan, and it is sufficiently shallow (maximum depth, 7 m; average, 4 m) that the biological processes in the sediment exert a significant effect on the aquatic environment. The temperature, pH, concentration of dissolved oxygen, and turbidity of the water column at the sampling site were 7°C, 8.1, 7.2 mg/liter, and 65 mg/liter, respectively. After the sampling, the sediment samples (depth, 0 to 10 cm) were cooled immediately

TABLE 1. Phylogenetic affiliations of 16S rRNA gene clones belonging to the domain *Bacteria*

Phylum	No. of clones	% of total	No. of types[a]
Proteobacteria			
Alpha class	2	1.8	2
Beta class	14	12.5	12
Gamma class	11	9.8	7
Delta class	26	23.2	20
Nitrospira	15	13.4	6
Acidobacteria	9	8.0	8
Bacteroidetes	7	6.3	6
Chlorobi	5	4.5	4
Actinobacteria	2	1.8	2
Cyanobacteria	1	0.9	1
Verrucomicrobia	1	0.9	1
Planctomycetes	3	2.7	3
Chloroflexi	8	7.1	8
OP8	1	0.9	1
WS3	2	1.8	1
Unidentified	4	3.6	4
Total	112	100	86

[a] An identical type was defined as a group of sequences with >97% similarity.

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on ice and stored at $-20°C$ for molecular analysis. The analytical data of the sediment were as follows: water content, 68.1%; pH 6.4; ignition loss, 18.1%; total carbon, 5.04%; total nitrogen, 0.58%; particle classification, silty clay (7% sand, 52% silt, 41% clay).

Phylogenetic analysis of bacterial diversity based on a 16S rRNA gene clone library. Total nucleic acids were extracted and purified from the sediment as described by Purdy et al. (37) and Koizumi et al. (27). The PCR amplification of 16S rRNA genes (1,175 bp, *Escherichia coli* positions 338 to 1513) from the purified genomic DNA was carried out with bacterial universal primers: mixtures of EUB338, EUB338I, EUB338II, and EUB338III designated by Daims et al. (9) for recovering almost all known bacterial lineages, and 1492R (28). The PCR conditions were as follows: initial denaturation at 95°C for 9 min, followed by 18 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min. To minimize the PCR bias (25, 46), the number of PCR cycles was decreased to 18 (43, 48). The purified rRNA gene fragments were cloned with a pT7 Blue T-vector kit (Novagen). The clonal DNAs were amplified from randomly selected recombinants by direct PCR with M13 primers (M4, 5′-GTTTTCCCAGTCACGAC-3′; RV, 5′-CAG GAAACAGCTATGAC-3′), purified with a MicroSpin S-400 HR column, and then used as templates for sequencing. Sequencing was performed with primer 907R (5′-CCGYCAAT TCMTTTRAGTTT-3′), a DTCS-Quick Start kit (Beckman), and a CEQ-2000 automated sequence analyzer (Beckman).

The sequences of all bacterial 16S rRNA gene clones with a range of about 500 to 600 bases were determined. All sequences were compared with those in the GenBank database (www.ncbi.nlm.nih.gov/BLAST) by using the BLAST program (2). To detect and omit chimeric DNAs, the CHECK-CHIMERA program (32) of the Ribosomal Database Project was used. Phylogenetic analysis was performed with the ARB program package (http://www.arb-home.de/). All clonal sequences and the reference sequences from the GenBank database were imported into a database of the ARB program. After automatic and manual sequence alignment, phylogenetic trees were constructed by the neighbor-joining method (41) and bootstrap analyses for 1,000 replicates were performed.

A total of 112 clones from the 16S rRNA gene library were analyzed in order to estimate the bacterial diversity in the freshwater sediment (Table 1). The sequence analysis grouped the clones into 86 distinct types (i.e., sequences with greater than 97% similarity were treated as identical). The coverage value derived from the equation described by Giovannoni (15) was 23.2%. On the basis of the phylogenetic analysis, the clonal sequences were affiliated with at least 10 classes of the domain *Bacteria*. The most dominant group of our clone library was allocated to the phylum *Proteobacteria* (47% of the total number of clones), and the dominant organisms within this class were in the delta, beta, and gamma classes (23.2, 12.5, and 9.8%, respectively). The second most dominant group of the clone library, represented by 15 clones (13.4%), was classified into the phylum *Nitrospira*. The other groups of the library were determined to be, in order of abundance, in the phyla *Acidobacteria*, *Chloroflexi*, *Bacteroidetes*, *Chlorobi*, *Planctomycetes*, *Actinobacteria*, *Verrucomicrobia*, and *Cyanobacteria*. Some clones affiliated with the candidate phyla WS3 and OP8,
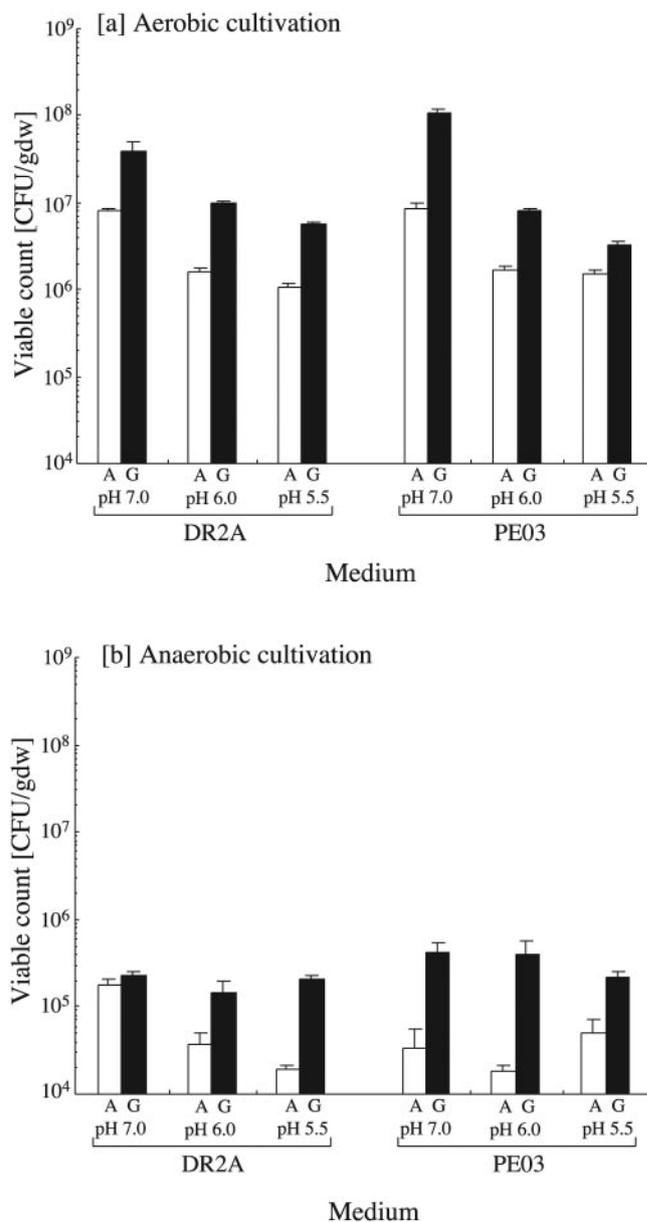

FIG. 1. Effects of medium composition, pH conditions, and gelling reagents on viable counts after 11 weeks of incubation under aerobic (a) and anaerobic (b) conditions. White and black bars represent CFU counts from agar-based (A) and gellan gum-based (G) media, respectively. The vertical bars indicate 1 SD from the mean.

which were represented solely by environmental clones (11, 19), were also observed.

Sequences allocated to the delta and beta groups of the phylum *Proteobacteria* (delta and beta *Proteobacteria*, respectively) have also frequently been retrieved from freshwater sediments in previous studies (33, 45, 50). In particular, delta *Proteobacteria* has been indicated as the representative bacterial lineage in benthic environments, since this group was more frequently recovered from sediments than from water columns, in which alpha, beta, and gamma *Proteobacteria*; *Bacteroidetes*; and *Actinobacteria* were observed as the dominant groups (18, 30, 45). This finding may be due to the oxidation-reduction
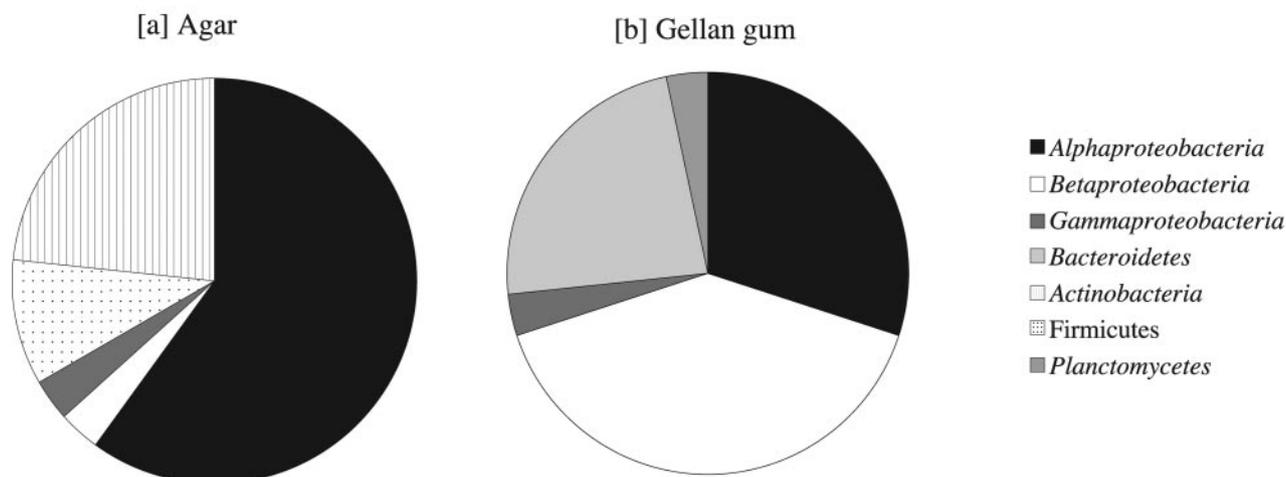
FIG. 2. Effects of gelling reagents (agar [a] and gellan gum [b]) on the phylogenetic distribution of microbes cultivated from freshwater sediment with PE03 medium (pH 7.0) over various bacterial lineages on the basis of 16S rRNA gene analysis.

potential gradient between the water and sediment environments. In fact, our study, as well as previous reports (29, 39, 45), frequently detected clones moderately related to the strict anaerobes, such as sulfate reducers (the genera *Desulfococcus*, *Desulfomonile*, and *Desulfonema*) and syntrophic bacteria (e.g., members of the genus *Syntrophus*), within delta *Proteobacteria* (supplemental Fig. A2 [all of the supplemental material cited in this report is available online at http://staff.aist.go.jp/tamaki-hideyuki/]).

There was no clone whose 16S rRNA gene sequence was 100% identical to those of known bacterial species. Only three clones had more than 97% sequence similarity to known bacterial species. One such clone, KTS75, was closely related to *Pelobacter propionicus* within delta *Proteobacteria* (supplemental Fig. A2) (45). The remaining two clones, KTS29 and KTS25, were affiliated with *Skeletonema pseudocostatum* within *Cyanobacteria* and *Novosphingomonas subarcticum* within alpha *Proteobacteria*. In addition, there were only three clones (KTS104, KTS6, and KTS9) having more than 95% sequence similarity to the previously described species (supplemental Fig. A1 and A3). Apart from the above-mentioned six clones, all of the remaining clones showed less than 95% 16S rRNA gene sequence similarity to any other identified species. The majority of the remaining clones were related to previously described uncultured environmental clone clusters, i.e., RBF8 within beta *Proteobacteria* (4), subclass 6 within *Acidobacteria*, and subclass 1 within *Chloroflexi* (19). Some other clones formed clusters (designated KTS II, KTS III, and KTS IV) with some previously known environmental sequences: KTS II within gamma *Proteobacteria*, KTS III within *Nitrospira*, and KTS IV within *Bacteroidetes* (supplemental Fig. A1 and A3). Moreover, some of the remaining clone sequences were not closely related to any of the previously published environmental sequences and thus formed independent unique clusters, designated KTS I and KTS V, which are affiliated with delta *Proteobacteria* and *Chlorobi*, respectively (supplemental Fig. A2 and A3[b]).

These results indicate that the bacterial community in the freshwater sediment of Lake Kasumigaura is remarkably di-

verse and is primarily composed of unknown bacterial species. Therefore, cultivation-based study was necessary to reveal the function of the unknown bacteria, as determined by 16S rRNA gene clone analysis.

Direct enumeration of microbial cells in sediment samples. Direct counting of visible cells in sediment samples was performed under an epifluorescence microscope with ethidium bromide staining, which allowed a significant reduction in the autofluorescence background derived from sediment particles. The number of microbial cells was calculated to be 8.37×10^9/g (dry weight) of sediment (standard deviation [SD] = 3.12×10^8 cells per g).

Cultivation of sediment microbes with agar- and gellan gum-based media. In this study, to capture a diverse array of microorganisms, we focused on three factors: constituents of the nutrient media (PE03 and DR2A), pH conditions (pHs 5.5, 6.0, and 7.0), and gelling reagents (agar and gellan gum). Moderately low-nutrient media DR2A and PE03 were used for cultivation. The composition of DR2A was as follows (per liter): 0.05 g each of yeast extract, peptone, acid hydrolysate of casein, dextrose, and soluble starch; 0.03 g each of dipotassium phosphate and sodium pyruvate; and 0.0024 g of magnesium sulfate. The composition of PE03 was as follows (per liter): 0.05 g each of sodium glutamate, sodium succinate, sodium acetate, yeast extract, Casamino Acids, sodium thiosulfate, and ammonium sulfate; 5 ml of basal salts solution (17); and 0.2 ml of vitamin solution (17). The pH values of these media were adjusted with 10 mM potassium phosphate buffer to 5.5, 6.0, and 7.0. As gelling reagents, gellan gum (Wako, Tokyo, Japan) and agar (Noble agar; Difco) were used for solidification of the media at final concentrations of 1.0 and 1.5%, respectively. The media were designated PE03-7G, PE03-6G, and PE03-55G for PE03 media solidified with gellan gum (pHs 7.0, 6.0, and 5.5, respectively). PE03 agar media (pH 7.0, 6.0, and 5.5) were designated PE03-7A, PE03-6A, and PE03-55A, respectively. In the same manner, the DR2A media were referred to as DR2A-7G, DR2A-6G, DR2A-55G, DR2A-7A, DR2A-6A, and DR2A-55A, respectively.

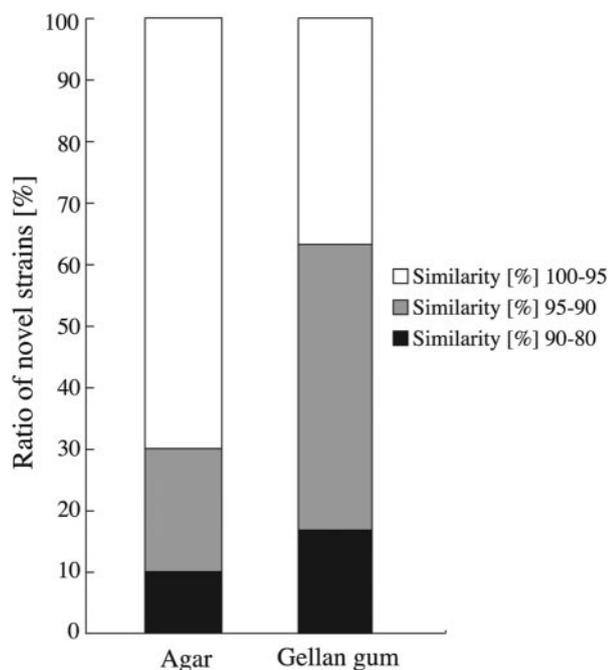Sediment samples (10 g) were suspended in sterile water and

FIG. 3. Effects of gelling reagents in PE03 medium (pH 7.0) on the cultivation of novel microbes from freshwater sediment under aerobic conditions. The similarity percentages shown are the 16S rRNA gene sequence similarities between the cultivated microbes and their closest relatives in the GenBank database.

diluted in 10-fold steps. A series of medium plates (12 different types, as described above) was inoculated with 100-μl aliquots from different dilutions and incubated at 20°C for 11 weeks in the dark under aerobic and anaerobic conditions. Anaerobic cultivation was performed with an AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan).

The number of CFU on all types of media continuously increased for more than 8 weeks. The viable counts shown in Fig. 1 were determined after 11 weeks of cultivation. Under aerobic conditions, viable counts from all of the types of gellan gum-based media used were 2.2 to 12.6 times greater than those from all of the agar media used (Fig. 1a). As regards the pH conditions, the viable counts tended to decrease as the pH decreased. The composition of the media had little influence on the viable counts. The maximum viable count was obtained on PE03-7G (gellan gum) and reached 1.10×10^8 CFU/g (dry weight) of sediment (SD = 1.14×10^7 CFU/g, 1.3% of the mean microscopically determined total cell count). This value was 12.6 times higher than the viable count, 8.68×10^6 CFU/g (SD = 1.23×10^6 CFU/g, 0.1% of the total cell count), as determined by using the same medium solidified with agar, PE03-7A. Likewise, under anaerobic conditions, the viable counts from all of the gellan gum media were also 1.3 to 21.3 times greater than those from all of the agar media (Fig. 1b). The effects of pH and the composition of the media on the viable counts were not remarkable. In general, the viable counts observed under anaerobic conditions were lower than those obtained under aerobic conditions by 2 to 3 orders of magnitude. Although the maximum viable count under anaer-

obic conditions was found on PE03-7G, the value was only 4.15×10^5 CFU/g (SD = 1.33×10^5 CFU/g).

In the previous studies of soil ecosystems, Sait et al. (40) reported only slight differences between the viable counts from gellan gum-based and agar-based media. Janssen et al. (20) demonstrated that the CFU counts obtained from gellan gum media were higher than those obtained from agar media. However, the differences in CFU counts between the two types of media in our study were much higher than those observed in their studies and were statistically significant ($P < 0.05$) on the basis of Student's *t* test except for the result obtained at pH 5.5 under anaerobic conditions. The use of gellan gum as a gelling reagent can be more effective than that of agar in improving the culturability of sediment microbes.

Phylogenetic distribution of cultivated sediment microbes. Identification of the microbes grown on the media was performed on the basis of the 16S rRNA gene sequences. Thirty colonies were randomly selected from all of the medium types with gellan gum, and their 16S rRNA gene sequences were determined. 16S rRNA genes were directly PCR amplified from single colonies grown on plates with bacterial universal primers 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (28). The 16S rRNA gene sequences determined were compared with those in the GenBank database by using the BLAST program (2). Furthermore, to investigate the effect of gelling reagents on the spectrum of culturable microbes, 30 colonies were also selected from the agar-based medium PE03-7A, which showed the highest CFU count among the agar-based media. The phylogenetic distributions of agar-cultured microbes were compared to those retrieved from the gellan gum-based medium PE03-7G, which gave the highest CFU count among the gellan gum-based media.

The cultivated microbes under aerobic conditions were affiliated with seven major bacterial lineages: alpha, beta, gamma, and delta *Proteobacteria*; *Firmicutes*; *Actinobacteria*; *Bacteroidetes*; and *Planctomycetes*. Growth of microbes affiliated with four to six major lineages was observed on all types of media. Although the differences in cultivable bacteria between those cultured on PE03 and DR2A media were not remarkable, the shifts to acidic pH (pH 5.5 and 6.0) led to the preferential growth of microbes classified into alpha *Proteobacteria* and *Firmicutes* (supplemental Fig. A4).

The differences between gelling reagents exerted a significant influence on the diversity of cultivable microbes in the freshwater sediment (Fig. 2). The randomly picked colonies from gellan gum medium PE03-7G were affiliated with alpha, beta, and gamma *Proteobacteria* (30.0, 40.0, and 3.3% of the total number of colonies selected from this medium, respectively), *Bacteroidetes* (23.3%), and *Planctomycetes* (3.3%), whereas those from agar medium PE03-7A belonged to alpha, beta, and gamma *Proteobacteria* (60.0, 3.3 and 3.3%), *Firmicutes* (10.0%), and *Actinobacteria* (23.3%). Thus, on agar plates, the microbes classified into alpha *Proteobacteria*, *Firmicutes*, and *Actinobacteria* accounted for 93.3% of the total isolates from PE03-7A. Although there was no statistical evidence because of the small sample size, such remarkable differences in microbial composition between agar- and gellan gum-based cultivations suggest that a gelling reagent has a

TABLE 2. Phylogenetic affiliations of microbes grown on PE03-7G (gellan gum) medium on the basis of 16S rRNA gene sequences by using the BLAST program in the GenBank database

Taxonomic group and strain	Closest species	Accession no.	Similarity (%)	Length (bp)
Alpha *Proteobacteria*				
PE70G13	*Hyphomicrobium zavarzinii*	Y14306	88	219
PE70G23	*Magnetospirillum gryphiswaldense*	Y10109	91	521
PE70G8	*Roseomonas gilardii*	AY150045	94	558
PE70G9	*Roseomonas gilardii*	AY150045	94	558
PE70G30	*Roseomonas gilardii*	AY150045	94	558
PE70G3	*Methylocystis parvus*	Y18945	96	560
PE70G16	*Novosphingobium subarcticum*	AY167828	98	593
PE70G25	*Novosphingobium subarcticum*	AY167828	99	591
PE70G24	*Agrobacterium sanguineum*	AB062105	99	589
Beta *Proteobacteria*				
PE70G26	*Gallionella ferruginea*	L07897	89	615
PE70G6	*Burkholderia glathei*	AY154379	90	561
PE70G19	*Propionivibrio pelophilus*	AF016690	91	553
PE70G15	*Paucimonas lemoignei*	X92555	91	526
PE70G17	*Paucimonas lemoignei*	X92555	92	561
PE70G5	*Herbaspirillum seropedicae*	AJ238361	92	553
PE70G20	*Caenibacterium thermophilum*	AJ512945	96	626
PE70G28	*Caenibacterium thermophilum*	AJ512945	96	626
PE70G14	*Leptothrix cholodnii*	X97070	96	560
PE70G7	*Leptothrix cholodnii*	X97070	97	561
PE70G22	*Leptothrix cholodnii*	X97070	97	612
PE70G2	*Thiobacillus denitrificans*	AJ243144	97	560
Gamma *Proteobacteria* PE70G27	*Thermomonas haemolytica*	AJ300185	97	617
Bacteroidetes				
PE70G4	*Flexibacter aggregans*	AB078038	87	560
PE70G11	*Flexibacter sancti*	AB078067	90	542
PE70G10	*Flexibacter sancti*	AB078067	91	561
PE70G12	*Flexibacter sancti*	AB078067	91	560
PE70G29	*Flexibacter sancti*	AB078067	91	561
PE70G18	*Hymenobacter actinosclerus*	Y17356	93	610
PE70G21	*Hymenobacter actinosclerus*	Y17356	93	622
Planctomycetes PE70G1	*Planctomyces limnophilus*	X62911	93	483

great influence on capturing a diverse array of sediment microbes.

Under anaerobic conditions, 28 or 29 of 30 colonies grown on each medium type were affiliated with the family *Clostridiaceae* within the phylum *Firmicutes* (data not shown).

Effects of the improved cultivation method on culturing of novel microbes. For each medium, the percentage of novel bacterial strains among selected microbes was determined. We considered those isolates whose 16S rRNA gene sequences were less than 95% similar to those of any known bacterial species to be novel microbes, at least at the species level. The percentage of novel bacterial strains obtained under anaerobic conditions was low, accounting for less than 13.3% of the total number of examined microbes cultivated on each type of medium (data not shown). Under aerobic conditions, the greatest number of strains that could be considered novel was retrieved from PE03-7G (gellan gum), and the percentage of novel microbes reached 63.3% of the total number of isolates examined on this medium (Fig. 3). These novel isolates consisted of 13 different phylotypes (i.e., strains with greater than 97% seqence similarity were treated as the same type), and they were classified as alpha and beta *Proteobacteria*, *Bacteroidetes*, and *Planctomycetes* (Table 2). On the other hand, the percentage of

novel microbes found on PE03-7A (agar) was 30% of the total number of colonies selected from the medium (Fig. 3). These novel strains consisted of only three different phylotypes, and they were affiliated with only two groups: alpha *Proteobacteria* and *Firmicutes* (Table 3). These results clearly indicate the effectiveness of using gellan gum as a gelling reagent instead of agar for the cultivation of novel microbes from freshwater sediment.

Of the novel strains grown on PE03-7G medium, the most abundant strains (seven) were affiliated with *Bacteroidetes* (Table 2). Of these seven strains, five (PE70G4, PE70G10, PE70G11, PE70G12, and PE70G29) were related to the genus *Flexibacter*, although they showed considerably lower similarities (87 to 91%) to any of the known bacterial species. The remaining two strains, PE70G18 and PE70G21, were related to *Hymenobacter actinosclerus*, with 93% 16S rRNA gene similarity. The second most abundant novel strains (i.e., six strains) retrieved from PE03-7G medium were affiliated with beta *Proteobacteria*. We found that three strains (PE70G5, PE70G15, and PE70G17) were distantly related to the family *Oxalobacteraceae*; one strain (PE70G5) was related to members of the genus *Herbaspirillum*, with 92% identity; and two strains (PE70G15 and PE70G17) were related to *Paucimonas lem-*

TABLE 3. Phylogenetic affiliations of microbes grown on PE03-7A (agar) medium on the basis of 16S rRNA gene sequences by using the BLAST program in the GenBank database

Taxonomic group and strain	Closest species	Accession no.	Similarity (%)	Length (bp)
Alpha *Proteobacteria*				
PE70A15	*Rhodoplanes elegans*	D25311	89	624
PE70A22	*Rhodoplanes elegans*	D25311	89	624
PE70A19	*Paracraurococcus ruber*	D85827	93	641
PE70A1	*Roseomonas gilardii*	AY150045	94	609
PE70A5	*Roseomonas gilardii*	AY150045	94	610
PE70A11	*Roseomonas gilardii*	AY150045	94	602
PE70A20	*Roseomonas gilardii*	AY150045	94	632
PE70A6	*Hyphomicrobium zavarzinii*	Y14306	94	348
PE70A10	*Methylosinus sporium*	Y18946	95	603
PE70A24	*Methylosinus sporium*	Y18946	96	620
PE70A3	*Methylosinus sporium*	Y18946	97	634
PE70A17	*Rhodobacter capsulatus*	D16427	97	608
PE70A18	*Microvirga subterranea*	AY078053	97	617
PE70A25	*Bradyrhizobium elkanii*	U35000	99	630
PE70A29	*Bradyrhizobium elkanii*	U35000	99	601
PE70A23	*Bradyrhizobium elkanii*	U35000	100	623
PE70A30	*Bradyrhizobium elkanii*	U35000	100	635
PE70A9	*Methylobacterium radiotolerans*	D32227	100	603
Beta *Proteobacteria* PE70A28	*Roseateles depolymerans*	AB003624	96	612
Gamma *Proteobacteria* PE70A8	*Pseudomonas putida*	D85999	99	626
Firmicutes				
PE70A13	*Fusibacter paucivorans*	AF050099	88	552
PE70A16	*Paenibacillus hongkongensis*	AF433165	95	595
PE70A14	*Bacillus cereus*	AE017013	99	506
Actinobacteria				
PE70A2	*Mycobacterium manitobense*	AY082001	98	616
PE70A7	*Mycobacterium manitobense*	AY082001	98	616
PE70A26	*Rhodococcus equi*	M29574	98	643
PE70A4	*Arthrobacter oxydans*	AJ243423	99	615
PE70A12	*Mycobacterium brumae*	AF480576	99	616
PE70A27	*Janibacter limosus*	Y08539	99	641
PE70A21	*Nocardia veterana*	AF430059	100	638

oignei, with 92% similarity. The growth of three strains (PE70G6, PE70G19, and PE70G26) that were affiliated with uncultured cluster RBF8 (4), representing a novel order within beta *Proteobacteria*, was also observed on PE03-7G. We also obtained a novel strain (PE70G1) belonging to the genus *Planctomycetes*. This strain was distantly related to *Planctomyces limnophilus*, with a 16S rRNA gene similarity of 93%. A novel strain affiliated with this class was also grown on DR2A-7G medium (data not shown). Although the species closest to this strain was *Gemmata obscuriglobus*, the similarity was very low (88%). We are currently performing continuous cultivation of these novel strains to further investigate their physiological features, which will facilitate a better understanding of their functional role in freshwater sediments.

Comparison of bacterial diversity between the 16S rRNA gene clone library- and culture-based methods. Modifications of either pH conditions or gelling reagents had a significant influence on the examination of viable counts and the spectrum of microbes cultivated from freshwater sediment. The shifts to acidic pH conditions (pHs 5.5 and 6.0) reduced the viable counts and led to the preferential growth of bacteria belonging to alpha *Proteobacteria*, *Actinobacteria*, and *Firmi-*

cutes, which were found to be insignificant groups by 16S rRNA gene clone analysis (supplemental Fig. A4). Under slightly acidic pH conditions, Janssen et al. (20) and Sait et al. (40) successfully cultivated globally distributed unknown bacteria affiliated with *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Verrucomicrobia* from soil samples. However, in this study, modification of the pH conditions appeared to be ineffective for increasing culturability and thereby at isolating novel bacterial strains from the freshwater sediment.

The preferential growth of bacteria belonging to alpha *Proteobacteria*, *Actinobacteria*, and *Firmicutes*, which were minor groups in the 16S rRNA gene clone library, was observed on the agar medium (Fig. 2). In contrast, the use of gellan gum medium led to the growth of a number of microorganisms belonging to beta *Proteobacteria* and *Bacteroidetes* (Fig. 2), which were more abundant in the clone library. In addition, 11 of 30 colonies selected from the gellan gum medium showed more than 95% sequence similarity to the 16S rRNA gene clones, while only 1 of 30 colonies from the agar medium showed such a sequence similarity.

The significant discrepancy between bacterial community compositions determined by culture-dependent and -indepen-

dent analyses has been revealed by many previous studies of a wide variety of natural ecosystems (10, 12–14, 24, 26, 36, 42, 44, 47). Our study clearly demonstrated that the use of gellan gum instead of agar medium would enable closure of the gaps between the two approaches. However, differences between the phylogenetic distributions of microbes shown by molecular and cultivation-based studies still remain and several explanations for these remaining differences appear plausible. First, a well-recognized primary reason for such differences is the bias associated with the use of molecular methods (12, 13, 24, 26, 36, 44, 47). Second, the anaerobic culture techniques used in this study were not suitable to cover anaerobes, which are thought to be quite abundant in freshwater sediment. Third, a percentage of the unknown bacteria in freshwater sediment may indeed be extremely difficult to cultivate in general. Recently, a number of researchers succeeded at increasing culturability and they subsequently cultivated hitherto uncultured bacteria by modifying the traditional cultivation techniques as follows: use of plant polysaccharides as an energy source (7), addition of signal molecules related to cell-cell communication (5, 6, 35), and effective use of background bacteria as aids to growth (23, 51). Our study strongly suggested that the gelling reagent used is an important factor in the successful cultivation of unknown bacteria. Certainly, the use of gellan gum alone is not sufficient for a culture-based study. However, the combination of the use of gellan gum and modifications of previously designed methods might lead to the cultivation of yet-to-be-isolated organisms and might also increase the opportunity to study bacterial diversity in a wide variety of ecosystems by culture-dependent analyses.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of both the clone library and culture collection have been submitted to public databases under accession numbers AB127608 to AB127929.

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