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Phylogeny of numerically abundant culturable anaerobic bacteria associated with  
degradation of rice plant residue in Japanese paddy field soil

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Abstract

Culturable anaerobic bacterial populations on rice plant residue (straw and stubble with roots) in paddy field soil were found on the order of  $10^9$  CFU (colony-forming unit) (g dry wt of plant residue)<sup>-1</sup>, and the percentages of spores were usually less than

1% of the total anaerobes. Anaerobic bacteria were isolated from each sample by picking up colonies on the roll tube agar used for the enumeration. The phylogenetic analysis of 47 isolates based on 16S rRNA gene sequences revealed that the composition of dominant culturable anaerobic bacteria on rice plant residue was rather simple. The most dominant group was closely related to the *Cellulomonas* species in the *Actinobacteria* phylum and accounted for more than 60% of the isolates for most of the samples. The second major group was also affiliated with the *Actinobacteria* phylum and tentatively named the “propionate-producing *Actinobacteria* group” because the strains in the group commonly produced propionate. Strains in the third group, the “*Prevotella*-like group”, were Gram-negative, strictly anaerobic rods and placed in the *Bacteroides* phylum with 16S rRNA gene similarities of 86-92% to the closest relatives. Some other strains belonging to *Betaproteobacteria* and the clostridial group were also isolated. Most of the strains affiliated to the clostridial group were isolated from the heat-treated samples. Some phenotypic characteristics of representative strains of each group are also described.

*Keywords:* CH<sub>4</sub> production; Rice field soil; Anaerobic bacteria; *Cellulomonas*;

Phylogeny based on 16S rRNA

## **1. Introduction**

Anoxic flooded paddy soil is one of the major sources of atmospheric methane [1-6]. Methane is produced by methanogenic archaea in anaerobic microbial ecosystems mainly from acetate or H<sub>2</sub> + CO<sub>2</sub> supplied by fermentative microbes in the ecosystem through degradation of organic matter [7-11]. In flooded paddy soil, the anaerobic microbial community, which consists of various microbes including methanogenic archaea, sulfate reducers, iron reducers, and various fermentative bacteria, develops, and degradation of organic matter coupled with various terminal electron-accepting processes proceeds actively [10-20].

Determination of methane emission rates from rice fields has been carried out extensively in various areas in the world, and it has been shown that addition of rice



straw to paddy soil results in a significant increase in the methane emission rates from the fields [3,6,21-23]. We determined variations and distributions of the methanogenic activity in Japanese paddy field soil for several years and showed that plant residue plowed into flooded soil maintained high methanogenic activity and significantly contributed to methanogenesis in the soil [24-28]. Glissmann et al. [29,30] showed that rice straw added to paddy soil was mainly colonized by hydrolytic and fermentative bacteria. These bacteria may release their fermentation products around plant residue and support methanogenesis in the soil. These findings indicate that plant residue in paddy soil serves as an important source of substrates or sites of methanogenesis in paddy soil.

Recent investigations of the microbial community in paddy soil have been performed extensively using cultivation-independent molecular techniques and cultivation-based techniques [10,12-15,31]. Cultivation-independent, 16S rRNA-dependent molecular and ecological investigations have revealed diversity in both archaeal and bacterial communities in anoxic paddy soil [10,14,17,31-33]. Weber

et al. [33] analyzed the bacterial community degrading rice straw under anoxic conditions with a molecular technique and showed the dominance of members of different clostridial clusters.

It has been well recognized that only a few microbes are culturable compared with the diversity really present in a natural habitat [14,34-36]. However, cultivation-based investigations of microbes have isolated many novel bacterial species, such as ultramicrobacteria belonging to the *Verrucomicrobales* [15], homoacetogenic bacteria [18], other fermentative bacteria [12,20], and sulfate-reducers [18,19], from anoxic paddy soil. This indicates the significance of cultivation-based investigation of microbes in anoxic paddy soil for further understanding of the microbial community. It has been shown that direct enrichment techniques select for populations that are fitter to the enrichment conditions used and grow faster. Thus, isolation of microbes from anoxic paddy soil has been often carried out using the highest positive dilutions of the most-probable-number (MPN) counting of microbes to exclude minor populations and isolate the most abundant culturable microbes [12,15,18,19].

Most of the investigations of anoxic paddy soil described above have been carried out using microcosms, which were cultivated with rice plants in laboratory scale containers with submerged soil [37], or soil slurries anaerobically incubated in flasks [7,38-40]. Investigations performed using actual rice fields are rare except for some reports on methanogens [41,42].

In this study, we analyzed the anaerobic microbial community associated with degradation of rice plant residue in Japanese paddy field soil by the cultivation-based method. In Japan, rice is a diet staple and paddy fields cover a vast area all over the country. We collected two types of rice plant residue (straw and stubble with roots) several times from the plow layer of paddy soil during the process of actual rice cultivation and enumerated anaerobic bacteria by colony counting them with the dilution culture techniques based on the anaerobic roll tube method [43,44]. Colonies grown on the agar medium were picked up randomly, and purified cultures of these strains were obtained. Phylogenetic affiliations of the isolates were determined by the analysis of 16S rRNA gene (16S rDNA) sequences, and some characteristics of

isolates were also determined. Distinctive features of the culturable anaerobic bacterial composition on rice plant residue in paddy field soil could be shown.

## **2. Materials and methods**

### *2.1. Rice field and sample collection*

All samples for the bacterial isolation were collected from the rice straw (RS) plot of rice fields in the Shonai Branch of the Yamagata Agricultural Experimental Station (Fujishima-machi, Yamagata, Japan) during the growing season of rice in 1993 and 1994. Rice straw has been annually applied ( $0.5 \text{ t ha}^{-1}$ ) to the RS plot, as well as inorganic fertilizers, for more than 20 years [25,26]. Characteristics of the soil were previously described [16,21,25,26].

The plot was continuously flooded from the beginning of May, just before transplanting of Japonica type rice (*Oryza sativa* cv. Haenuki), until the end of June. After the rice plants reached the maximum tiller number stage in late June, the field was drained for about 10 days as midseason drainage. Then, the field was subjected to

intermittent irrigation until the water used for flooding was drained in the middle of September. Rice straw cut into pieces (about 2 cm) is applied to the soil surface in the autumn after harvesting the rice and plowed into the soil before flooding of the next growing season. Stubble of rice plants left in the field with roots after harvest was also plowed into the soil together with rice straw. The “rice straw” samples used in this investigation contained stems, leaf shades, and leaf sheaths of rice plants. Above-ground parts of rice plants were cut off from stubble collected from soil, so that the “stubble” samples contained only basal parts of stems and leaf sheaths of rice plants as well as roots.

## *2.2. Sample preparation and bacterial isolation*

The plant residue samples for enumeration and isolation of anaerobic bacteria were collected from the plow layer of the field during the growing season. After soil adhering to rice plant residue collected from paddy soil was washed off several times with the anoxic diluent [16,24,25], the samples were cut into pieces and washed

several times again. The washed samples (5 g [wet weight]) were added to 45 ml of the anoxic diluent and homogenized by a Waring blender (10,000 rpm, 10 min) under N<sub>2</sub> gas, followed by consecutive 10-fold dilutions with the anoxic diluent under N<sub>2</sub> gas flow. Soil samples for enumeration of anaerobic bacteria were collected using core samplers (5 cm in diameter) from a depth of 0-10 cm of the plow layer of the field at the same time and diluted in the same way as described previously [25].

Enumeration and isolation of anaerobic bacteria were carried out with the anaerobic roll tube method [43-45] using oxygen-free N<sub>2</sub> 95%-CO<sub>2</sub> 5% mixed gas as headspace. Each diluted sample (0.3 ml) was inoculated in triplicate into PY agar medium supplemented with 0.25 g l<sup>-1</sup> each of glucose, cellobiose, maltose, and soluble starch (PY4S agar) [44, 45]. PY agar medium contained (l<sup>-1</sup>) 75 ml of salt solution I, 75 ml of salt solution II, 10 g of Trypticase (BBL, Cockeysville, MD.), 5 g of yeast extract (Difco Laboratories, Detroit, MI), 1 mg of resazurin-Na, 0.2 g of Na<sub>2</sub>CO<sub>3</sub>, 0.3 g of L-cysteine • HCl • H<sub>2</sub>O, and 15 g of Agar (Difco Laboratories). The salt solution I contained 6 g l<sup>-1</sup> of K<sub>2</sub>HPO<sub>4</sub>. The salt solution II contained (l<sup>-1</sup>) 6 g of KH<sub>2</sub>PO<sub>4</sub>, 12 g of

$(\text{NH}_4)_2\text{SO}_4$ , 12 g of NaCl, 1.2 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 1.2 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ . The medium was adjusted to pH 7.3 with 1 M NaOH.

Diluted samples treated at 80°C for 10 min were also used in the same way to enumerate and isolate bacteria present as spores in the samples. The number of colonies that appeared on the agar medium during 12 days of incubation at 30°C was counted to determine the number of culturable anaerobic microbes in the samples. The period of incubation was determined from several preliminary experiments, which showed that the number of colonies almost reached its maximum during about 10 days of incubation. Thereafter, 15-20 colonies were picked up at random for every sample from the roll tube agar. The second highest dilutions (usually  $10^{-6}$  dilutions) of positive tubes were usually selected for the isolation, since the number of colonies on the roll tube agar of the highest dilutions was too few to isolate enough colonies. The isolates were purified by repeating the colony isolation by the anaerobic roll tube method, and the strains finally purified were used in this study. Colonies were also isolated from the agar medium inoculated with the heat-treated samples in the same way. The purity of

the isolates was confirmed by uniform colony morphology on the roll tube agar and cellular morphology with Gram staining, as well as observations by phase-contrast microscopy. The purified strains were maintained in the PY4S agar slants with the mixed gas in the headspace.

### *2.3. Phenotypic characterization of isolates*

Oxidase activity was examined using cytochrome oxidase test strips (Eiken Chemicals Co. Ltd., Tokyo, Japan), and catalase activity of cells was tested by the O<sub>2</sub> generation in 3% H<sub>2</sub>O<sub>2</sub>. The nitrate-reducing ability was examined by cultivating the strains in the PY liquid medium supplemented with NaNO<sub>3</sub> (2 g l<sup>-1</sup>) and glucose (2 g l<sup>-1</sup>) [44]. To determine fermentation products from glucose, the strain was cultivated in the PY liquid medium supplemented with glucose at 10 g l<sup>-1</sup> (PYG liquid medium) under the mixed gas. Utilization of different carbohydrates was tested in the PY liquid medium with each substrate added at 10 g l<sup>-1</sup>. Growth in each medium was monitored by measurement of the optical density at 660 nm with a spectrophotometer (Hitachi



U-1000, Katsuta, Japan). Growth in the medium without supplements was used as controls. Spore formation was examined by the observation of cell morphologies with Gram staining or the growth in the PYG liquid medium of the cells with treatment at 80°C for 10 min after inoculation. Growth of the strains under the aerobic condition was examined by plate culture on nutrient agar (Nissui Pharmacy Co. Ltd., Tokyo, Japan) and PYG agar medium. Cultivation temperature was 30°C for all tests.

#### *2.4. Analytical methods*

Volatile fatty acids and alcohols were analyzed with a gas chromatograph (Hitachi G-5000 or 263-30, Katsuta, Japan) equipped with a flame ionization detector with N<sub>2</sub> as the carrier gas, as described previously [46]. Non-volatile fatty acids and formate were analyzed with a high-performance liquid chromatograph (Shimadzu LC-10AD, Kyoto, Japan) equipped with a CDD-6A electroconductivity detector, a Shimpack SPR-H organic acid column, and a SCR-102H Guard column. The elutant was 5 mM *p*-toluenesulfonic acid with a flow rate of 0.8 ml/min and an injection volume of 20 µl.

The column temperature was 40°C. Gas samples were taken from the headspace of culture tubes with a pressure-lock syringe and analyzed with a gas chromatograph (Hitachi 163, Katsuta, Japan) equipped with a thermal conductivity detector using argon as a carrier gas, as described previously [46].

## 2.5. 16S rDNA sequencing and phylogenetic analysis

Cells cultivated in PYG liquid medium were collected by centrifugation and used for DNA extraction. The cells resuspended in 50 µl of distilled water were treated with three consecutive freeze-thaw cycles and then lysed by the addition of 40 µl of BL buffer (40 mM Tris-HCl [pH 8.0], 1% [v/v] Tween 20, 0.5% [v/v] Nonidet P-40, 1 mM EDTA) and 10 µl of Proteinase K solution (1 mg/ml; Boehringer Mannheim, Mannheim, Germany) followed by incubation at 60°C for 25 min and 95°C for 5 min.

16S rDNAs were PCR-amplified from DNA extracts with the 27f (5'-AGAGTTTGATCCTGGCTC-3'; *Escherichia coli* positions 8 to 27) and 1492r (5'-GGCTACCTTGTTACGACTT-3'; *E. coli* positions 1510 to 1492) primers [47], and

were purified by polyethylene-glycol precipitation. PCR-amplified 16S rDNA was sequenced using the Thermo sequenase cycle sequencing kit (Amersham Pharmacia Biotech, Cleveland, USA) and a DNA sequencer model 4000L (LI-COR, Lincoln, USA).

Multiple alignments of the sequences obtained and reference sequences in the GenBank database with the BLAST program [48] were performed, and a phylogenetic tree with the neighbor-joining method [49] was constructed using the CLUSTAL W program [50]. All gaps and unidentified base positions in the alignment were excluded before calculations.

#### *2.6. Nucleotide sequence accession number*

Accession numbers of the sequences determined in this study are AB078817 to AB078861, AB084626, and AB084627.

### **3. Results**

### *3.1. Seasonal variations in the number of anaerobic bacteria*

Enumeration of anaerobic bacteria on the two types of rice plant residue, straw and stubble with roots, in the RS plot was carried out in 1993 and 1994. In 1993, the enumeration was performed twice, on May 30 (early flooding period) and August 7 (intermittent irrigation period). In 1994, the enumeration was performed once a month from May to August. In the four sampling times in this year, the first two samplings were during the flooded period of the field and the latter two were during the intermittent irrigation period.

The number of culturable anaerobic bacteria was at the order of  $10^9$  CFU (colony-forming unit) (g dry wt of plant residue)<sup>-1</sup> except for some samples (Table 1). In 1994, the counts from the stubble samples decreased distinctly with the progress of the growing season, while those from the straw samples did not change much. The number of spores was much lower than the number of total anaerobic bacteria, although it increased soon after midseason drainage at the end of July for both samples.

The number of culturable anaerobic bacteria in the soil samples collected at the same time was found on the order of  $10^6$  CFU (g dry wt of soil)<sup>-1</sup> for most of the samples, and the percentages of spores were considerably higher than those of plant residue (Table 1).

Colonies on the roll tube agar inoculated with the plant residue samples were picked up, and purified strains were obtained. For 1993, a total of 40 strains from the untreated samples of the both sampling times and a total of 9 strains from the heat-treated samples on August 7 were finally obtained. Then, a total of 21 strains were selected as representatives of the bacterial groups classified by aerobic growth ability and cellular morphology. Among the isolates in 1994, all 26 strains (16 from straw and 10 from stubble) isolated from the untreated samples obtained from flooded soil on June 16 were used without any selection. Strains isolated from the straw samples were designated "W" strains, and those from the stubble samples designated "K" strains. Strains isolated from the heat-treated samples were named "80" strains (Table 2). Phylogenetic analysis and some phenotypic characterizations, including

catalase activity, oxidase activity and fermentation products from glucose, were carried out on all 47 strains selected.

### 3.2. Phylogenetic analysis of isolates

Table 3 shows the 16S rDNA-based phylogenetic placement of all 27 strains derived from the rice straw samples. The placement was as follows: two strains, *Betaproteobacteria* class of the *Proteobacteria* phylum; one strain, *Bacteroides* phylum; 22 strains, *Actinobacteria* phylum (high G+C gram-positive bacterial group); two strains, *Firmicutes* phylum (low G+C gram-positive bacterial group) [51].

Table 4 shows the phylogenetic placement of all 20 strains from the rice stubble samples. Eight strains belonged to the *Bacteroides* phylum, 10 strains were grouped in the *Actinobacteria* phylum, and two strains were in the *Firmicutes* phylum.

Figure 1 is the 16S rDNA-based phylogenetic tree showing the relationship of all 47 strains analyzed in this investigation.

Representative strains were selected for each phylogenetic group and some

additional phenotypic characterizations were performed. Detailed phylogenetic data and the phenotypic characteristics of the representative strains were as described below.

### 3.3. Strains isolated from the rice straw samples

#### (i) *Betaproteobacteria* group

Strains W5 and WB5 made a tight cluster with a 99.7% similarity of 16S rDNA to each other and belonged to the *Betaproteobacteria* class. Strain W5 was isolated in May of 1993, and one more morphologically similar strain was isolated from the same sample (Table 2). Strain WB5 was isolated in June of 1994. The closest known relative of both strains is *Chromobacterium vilaceum* [52] (Table 3). Both strains are Gram-negative, strictly anaerobic, curved rods (Fig. 2A), and the main fermentation products from glucose of the strains are shown in Table 5.

#### (ii) *Bacteroides* group

Strain WB4 isolated in June of 1994 belonged to the *Bacteroides* phylum. The strain

is strictly anaerobic, and cells are Gram-negative rods (Fig. 2B). The closest previously described relative of the strain is *Bacteroides fragilis* [53] (Table 3). The main products from glucose are acetate and propionate (Table 5).

(iii) *Actinobacteria* group

(a) *Cellulomonas*-like group

Eighteen strains from the straw samples were considered close to *Cellulomonas* species in the *Actinobacteria* phylum [54] and tentatively named the “*Cellulomonas*-like group” (Table 3). Since another four (in May) and seven (in August) strains with a quite similar cellular morphology were also included in the isolates in 1993 (Table 2), 70-80% of the isolates from the straw samples in 1993 were considered to belong to this group. Furthermore, 14 strains out of 16 isolated in June of 1994 were found to belong to this group.

All strains in the group are facultatively anaerobic, Gram-positive, slender, irregular rods with some straight or slightly curved cells (Fig. 2C, D). Fourteen strains in this group had similarity of 16S rDNA higher than 99% to each other and were



closely related to *Cellulomonas cellasea*. Other strains were assigned to near other *Cellulomonas* species [54,55] (Table 3). When anaerobically cultivated in PYG liquid medium, these strains produced formate, acetate, lactate, and ethanol. Anaerobic hydrolysis of cellulose powder by these strains could not be confirmed (Table 5).

(b) Propionate-producing *Actinobacteria* group

Three strains (W1, Wd, and Wf) were grouped and closely related to a clone SJA-181 derived from an anaerobic trichlorobenzene-transforming microbial community with the 16S rDNA similarities of 98.2 -98.7% [56]. These strains are facultatively anaerobic, Gram-positive, irregular rods. Cells of these strains are slightly smaller than those of *Cellulomonas*-like strains described above, and the two groups are morphologically distinguished each other (Fig. 2E). The closest previously described relative of the group is *Micropruina glycogenica* [57] (Table 3). These strains commonly produced propionate as well as other fatty acids (Table 5) and, thus, the group was tentatively referred to as the “propionate-producing *Actinobacteria* group”. Two strains other than Wf grew only weakly in PYG liquid medium under the

anaerobic condition, while strain Wf showed a good growth.

(c) Other strains

Strain W7 is a facultatively anaerobic bacterium, and cells are Gram-positive, irregular rods. The strain produced acetate, lactate, and ethanol from glucose and was closely related to *Microbacterium lavaniformans* [58] (Table 3).

(iv) Clostridial group

Two strains isolated from the heat-treated samples on August in 1993 are strictly anaerobic bacteria and both belong to the clostridial group in the *Firmicutes* phylum. Cells of strain 80Wc are Gram-positive, thin, spore-forming rods (Fig. 2F, Table 5), and the strain was related to *Clostridium populeti* [59] (Table3). Cells of strain 80Wd are Gram-positive, spore-forming rods. The main products of the strain from glucose were acetate, ethanol, and H<sub>2</sub>, and the strain was related to *Clostridium josui* [60] (Table 3). Although the phylogeny of other two isolates from the same heat-treated sample was not determined, they resembled strain 80Wd in both cellular morphology and fermentation products (Table 2).

### 3.4. Strains isolated from the stubble samples

#### (i) *Prevotella*-like group

Eight isolates out of 10 from the stubble samples in June of 1994 were found to belong to the *Bacteroides* phylum. Since the closest known relatives of the group belonged to the genus *Prevotella* [61] [Table 4], the group was tentatively named the “*Prevotella*-like group”. These strains were distinguished into the *Prevotella*-like group I (KB1, KB7, KB9, and KB12) and the *Prevotella*-like group II (KB3, KB10, KB11, and KB13) (Table 4). The similarity of 16S rDNA between the two groups was 89.3% when the value was calculated using strains KB7 and KB3 as representatives of each group (Fig. 1).

The four strains in the *Prevotella*-like group I are strictly anaerobic, Gram-negative rods (Fig. 2G, Table 5). The levels of similarity among strains KB7, KB9, and KB12 were about 99%, and strain KB1 was placed at a slight distance from the three strains (similarity of 97.3-97.5%). Although the four strains were most closely related to

*Prevotella* species, the levels of 16S rDNA similarity were rather low (Table 4). These strains produced acetate, malate, and succinate from glucose and used xylan for growth (Table 5).

The four strains in the *Prevotella*-like group II are also strictly anaerobic, Gram-negative rods growing in long filaments (Fig. 2H, Table 5). The similarities of 16S rDNA among the strains were 98.3-99.2%. These strains were only distantly related to *Prevotella* species (Table 4). These strains grew only poorly in PYG liquid medium and produced small amounts of propionate in addition to other fatty acids. They also utilized xylan (Table 5).

(ii) *Actinobacteria* group

(a) *Cellulomonas*-like group

Seven strains from the stubble samples were assigned to close to the genus *Cellulomonas* in the *Actinobacteria* phylum and grouped in the *Cellulomonas*-like group together with the isolates from the straw samples (Fig. 1). The characteristics of the strains in this group are the same as those of the *Cellulomonas*-like group

described above for the isolates from the straw samples (Fig. 2I, Table 5). Since one other (in May) and eight (in August) strains with a quite similar cellular morphology were isolated in 1993 from the stubble samples (Table 2), six out of 10 strains in May and all nine strains in August were deduced to be assigned to this group.

(b) Propionate-producing *Actinobacteria* group

Two strains (K2 and K5) were grouped in the propionate-producing *Actinobacteria* group together with the three isolates from the straw samples described above (W1, Wd, and Wf) (Table 4, Fig. 1). Both strains were isolates in May of 1993, and another morphologically similar strain was included in the isolates at that time (Table 2). The closest previously described relative for strain K2 was *M. glycogenica*, and it was *Microthricus phosphovorus* [62] for strain K5. The growth of the two strains in PYG medium was rather poor, and the other characteristics are similar to those described for the same group from the straw samples (Table 5).

(c) Other strains

Strain K4 is a facultatively anaerobic bacterium with cells of Gram-positive,

irregular rods. The closest relative of strain K4 was *Propionibacterium acnes* [63] (Table 4). The strain produced large amounts of acetate and propionate with lactate as a minor product. Although the characteristics of the isolates resemble the *Cellulomonas*-like group or the propionate-producing *Actinobacteria* group described above, the cellular morphology of strain K4 is distinct from those of the other two groups (data not shown).

(iii) Clostridial group

Strains KB6 and 80Kb were assigned to the clostridial group. Strain KB6, the sole strain that belonged to the clostridial group in the isolates from the untreated samples, is a strictly anaerobic bacterium with cells of Gram-positive, spore-forming rods. The closest known relative of strain KB6 was *Clostridium butyricum* [59] (Table 4). The strain, however, was more closely related to the xylanolytic strain RXyl1 isolated by the enrichment culture from the anoxic slurry of rice field soil with a similarity of 99.1% [20]. Strain KB6 produced acetate, butyrate, lactate, succinate, ethanol, and H<sub>2</sub> from glucose, which is consistent with strain RXyl1.

Strain 80Kb was isolated from the heat-treated sample in August of 1993. The strain is also a strictly anaerobic bacterium with cells of Gram-positive, spore-forming rods. The strain produced formate, acetate, lactate, succinate, ethanol, and H<sub>2</sub> from glucose. The closest relative of the strain was *Clostridium leptum* [59] with a rather low level of 16S rDNA similarity (Table 4). Although the phylogeny of the four other strains isolated from the same heat-treated sample was not determined, they resembled strain 80Kb in both cellular morphology and fermentation products from glucose (data not shown) (Table 2).

#### **4. Discussion**

Plant materials generally contain cellulose, hemicellulose, and lignin as the main components and it is reported that they comprise 48.8%, 20.9%, and 20%, respectively, of rice straw [64]. For the RS plot used in this study, rice straw has been plowed into paddy soil every year as well as rice stubble with roots. Thus, hydrolytic and fermentative anaerobic bacteria decomposing these polymers should have been

enriched in soil of the field. Kaku et al. [25] investigated the decomposition process of rice straw plowed into soil of the RS plot and indicated that rice straw was decomposed and broken down to pieces rapidly during the continuously flooded period from early May to late July. Thereafter, the decomposition rate became much slower, and small pieces, usually too small and thin to collect, remained in soil to be decomposed even at the time of harvest [65]. Stubble samples contained firmer and tougher parts than rice straw samples, and its decomposition rate seemed to be much slower than that of rice straw. We often observed stubble with rice roots left in paddy soil without distinct breakdown even late in the growing season.

Kaku et al. [16] enumerated the number of culturable anaerobic bacteria in rice plant residue in the RS plot by the anaerobic roll tube method using PY4S agar. The number was often more than 100 times higher than those in soil samples of the same paddy field (dry weight basis), and they suggested that rice plant residue served as the main habitat for fermentative microbes in the paddy soil. Similar results were also obtained in this study by the enumeration of anaerobic bacteria in plant residue as well



as soil samples collected from the RS plot.

All strains used in this study were derived from the colonies picked up at random from the sub-terminal positive tubes of the dilution series without any selection. Thus, the strains are thought to represent the dominant culturable anaerobic bacterial species present in plant residue. We considered that the strains isolated in May of 1993 and June of 1994 represent the groups involved in the initial decomposition of rice plant residue in anoxic soil and those isolated in August of 1993 represent the groups responsible for the final decomposition.

The phylogeny of the isolates determined based on 16S rDNA indicated that the dominant culturable anaerobic bacterial composition on rice plant residue was quite simple. The strains closely related to *Cellulomonas* species, the *Cellulomonas*-like group, were isolated from both samples at all three sampling times. The number of strains in this group was 60-100% of the isolates for all samples except the stubble samples in June of 1994. This indicates that the group is the dominant culturable bacteria on both types of rice plant residue irrespective of the period. *Cellulomonas*

species are cellulolytic and facultatively anaerobic bacteria, which have been isolated mainly from soil [54]. Several MPN bacterial isolates closely related to *C. fermentans* were also isolated from anoxic paddy soil in laboratory scale containers [14]. The strains examined in the present study reduced nitrate to nitrite and produced formate as a fermentation product. These results suggest that the bacterial group closely related to *Cellulomonas* species may play an important role in the decomposition of rice plant residue and the reduction process in flooded paddy soil.

Another main group affiliated with the *Actinobacteria* phylum, the propionate-producing *Actinobacteria* group, was isolated from both samples and both sampling times in 1993. A strain grouped with *M. phosphovorius* was also included in the MPN isolates from a microcosm of anoxic paddy soil described above (the similarity of 16S rDNA, 94.6%) [14]. The characteristics of the strain coincide with those of our isolates. Thus, it is suggested that the group is one of the ubiquitous anaerobic bacterial groups in anoxic paddy soil. Although all strains in the group produced propionate, the 16S rDNA similarities of the strains with the

*Propionibacterium* species [63] were 90.5% at most. Further characterization of these strains will be presented elsewhere.

All except two strains isolated from the stubble samples in June of 1994 appeared to belong to the *Prevotella*-like group in the *Bacteroides* phylum. The result indicates that the population of the group on the stubble samples at this time was significantly high. Since the strains in this group were not isolated from rice straw or at other sampling times, the group should be one of the major groups responsible for the initial decomposition of rice stubble or roots in anoxic flooded soil. The species in the closest genus *Prevotella* are known as xylan- and pectin-degrading strictly anaerobic bacteria present in the rumen [66]. Both *Prevotella*-like groups in this study were also able to utilize xylan, the main component of hemicellulose, for growth. Although the similarities of 16S rDNA indicate that the strains in the groups are phylogenetically distant from any known species, it is probable that the groups are responsible for the decomposition of hemicellulose of plant residue in paddy soil.

In the minor groups from the rice straw samples during the flooding period, the

strains affiliated with *Betaproteobacteria* were isolated in both years, suggesting that the bacterial group is one of the major groups involved in the initial decomposition of rice straw. The strain in this group produced a significant amount of formate from glucose and had nitrate-reducing ability. Another minor group, strain WB4 isolated in June of 1994, was only distantly related to the previously known species *Bacteroides fragilis*, one of the major anaerobic bacterial species in the intestinal tract of humans [53]. Further investigations to characterize these strains seem to be valuable to analyze the anaerobic bacterial community in anoxic flooded soil and to describe an anaerobic bacterial new species.

Weber et al. [33] used the molecular techniques to show the dominance of members of different clostridial clusters in the bacterial community degrading rice straw under anoxic conditions. In our study, the bacterial counts obtained from the heat-treated samples of plant residue were less than 1% of the total anaerobes during the flooded period, and most of the strains belonging to the clostridial group were isolated from the heat-treated samples, although the percentages of spores of soil

samples were much higher than those of plant residue. This finding indicates that bacteria belonging to the clostridial group are not dominant in the culturable bacteria on rice plant residue under the practice of actual rice cultivation, although investigations using other cultivation conditions including different compositions of media should be carried out to confirm the finding. Growth rates of the clostridial strains in PYG liquid medium were much higher than those of other major groups isolated in this study. Thus, if the enrichment procedure was used for the isolation directly without suitable dilution steps, it might be possible that the clostridial group outcompeted the major slow-growing bacterial species.

The characteristics of the three major groups isolated from plant residue (the *Cellulomonas*-like, propionate-producing *Actinobacteria*, and *Prevotella*-like groups) seem to coincide well with the properties of the samples used for isolation. Two groups (the *Cellulomonas*-like and *Prevotella*-like groups) are candidates for decomposers of cellulose or hemicellulose in plant residue. Many of the strains in the latter two groups (the propionate-producing *Actinobacteria* and *Prevotella*-like groups) grew only

poorly and produced propionate together with other fatty acids including acetate. Since propionate is an important intermediate in anaerobic digestion of organic matter [20,46], it is important to investigate the propionate formation as well as the optimum growth condition of these strains for further analysis on the anaerobic microbial community in paddy soil.

The strains in the *Cellulomonas*-like group as well as those in *Betaproteobacteria* produced formate as a fermentation product. Thus, the major anaerobic bacterial groups on rice plant residue seem to support growth of methanogens with formate or acetate, but not with H<sub>2</sub>, since only one out of 44 strains from the untreated samples produced H<sub>2</sub> as a fermentation product. In contrast, all clostridial isolates produced H<sub>2</sub>.

The results suggested that many culturable, but not yet described, bacterial species exist on plant residue in paddy soil. Growth of most of the bacterial strains investigated in this study was slow and weak on PY4S or PYG medium. We used PY4S agar as the sole medium under the fixed condition for the isolation. It is most probable that many other new anaerobic bacterial species will be isolated from the

microbial community in paddy soil by using various other media or cultivation conditions including the incubation temperature.

We did not count total bacterial populations directly in the plant residue samples used for the isolation. Further, we did not compare the culturable populations with those based on the 16S rDNA sequences recovered directly from the same samples. Thus, the dominance of the major groups of the isolates in the real plant residue should be further confirmed by comparing the populations obtained by these methods. In addition, since the sampling time was restricted, it is necessary to further analyze the changes in the population on plant residue during the decomposition and the differences in the population depending on the types of plant residue.

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Table 1. Numbers of anaerobic bacteria on rice plant residue (straw and stubble with roots) and in soil enumerated by the anaerobic role tube method

Date	Anaerobic bacteria			Spores of anaerobic bacteria <sup>a</sup>		
	CFU (g dry wt) <sup>-1</sup>			CFU (g dry wt) <sup>-1</sup>		
	Straw	Stubble	Soil	Straw	Stubble	Soil
1993. 5.30	$0.86 \times 10^9$	$1.40 \times 10^9$	— <sup>b</sup>	—	—	—
8.7	$2.00 \times 10^9$	$2.50 \times 10^9$	$5.97 \times 10^6$	$1.40 \times 10^7$ (0.70) <sup>c</sup>	$1.0 \times 10^7$ (0.40)	$3.69 \times 10^6$ (61.8)
1994. 5.27	$5.87 \times 10^9$	$3.83 \times 10^9$	$7.53 \times 10^6$	$2.53 \times 10^7$ (0.43)	$2.69 \times 10^7$ (0.70)	$8.27 \times 10^6$ (110)
6.16	$4.42 \times 10^9$	$3.84 \times 10^9$	$11.8 \times 10^6$	$1.83 \times 10^7$ (0.41)	$3.44 \times 10^7$ (0.90)	$6.87 \times 10^6$ (58.2)
7.12	$3.47 \times 10^9$	$1.86 \times 10^9$	$7.07 \times 10^6$	$6.61 \times 10^7$ (1.90)	$17.3 \times 10^7$ (9.30)	$1.94 \times 10^6$ (27.4)
8.16	$4.19 \times 10^9$	$0.61 \times 10^9$	$4.25 \times 10^6$	$7.18 \times 10^7$ (1.71)	$8.50 \times 10^7$ (13.9)	$1.81 \times 10^6$ (42.6)

<sup>a</sup> Homogenized samples diluted 10-fold were treated at 80°C for 10 min and inoculated into the medium.

<sup>b</sup> Not determined.

<sup>c</sup> Values in parentheses indicate the percentages of cells present as spores in the anaerobic bacteria.

Table 2. Number of strains isolated from rice plant residue in paddy soil and investigated in this study

Date	Source	Number of strains isolated	Number of strains analyzed	Strains analyzed <sup>a</sup>
1993.5.30	Straw	11	6	W1, W2 (3), W5 (1), W6, W7, W10 (1)
	Stubble	10	8	K2, K3, K4, K5 (1), K6, K8 (1), K10, K12
8.7	Straw	10	3	Wa (7), Wd, Wf
	Stubble	9	1	Ke (8)
	Heat-treated straw	4	2	80Wc, 80Wd (2)
	Heat-treated stubble	5	1	80Kb (4)
1994.6.16	Straw	16	16	WB2, WB4, WB5, WB6, WB7, WB8, WB9, WB10, WB11, WB12, WB13, WB14, WB15, WB16, WB17, WB18
	Stubble	10	10	KB1, KB3, KB6, KB7, KB8, KB9, KB10, KB11, KB12, KB13
Total		75	47	

<sup>a</sup> Numbers in parenthesis indicate the number of other isolates having a similar cellular morphology with the same Gram-staining reaction and the same growth behavior under the aerobic condition.

Table 3. Phylogenetic affiliations of strains isolated from rice straw in paddy soil

Affiliation	Group	Strain	Closest relative	% Similarity	Accession No.
<i>Betaproteobacteria</i>	<i>Betaproteobacteria</i>	W5	<i>Chromobacterium violaceum</i>	93.9	AB078837
		WB5	<i>Chromobacterium violaceum</i>	94.1	AB078843
<i>Bacteroides</i>	<i>Bacteriodes</i>	WB4	<i>Bacteroides fragilis</i>	86.1	AB078842
<i>Actinobacteria</i>	<i>Cellulomonas</i> -like	W2	<i>Cellulomonas cellasea</i>	97.3	AB078836
		W10	<i>Cellulomonas cellasea</i>	98.4	AB078840
		WB6	<i>Cellulomonas cellasea</i>	98.4	AB078844
		WB7	<i>Cellulomonas cellasea</i>	97.9	AB078845
		WB8	<i>Cellulomonas cellasea</i>	98.1	AB078846
		WB9	<i>Cellulomonas cellasea</i>	98.5	AB078847
		WB10	<i>Cellulomonas cellasea</i>	98.4	AB078848
		WB11	<i>Cellulomonas cellasea</i>	97.3	AB078849
		WB12	<i>Cellulomonas cellasea</i>	98.4	AB078850
		WB13	<i>Cellulomonas cellasea</i>	98.4	AB078851
		WB14	<i>Cellulomonas cellasea</i>	98.4	AB078852
		WB15	<i>Cellulomonas cellasea</i>	98.5	AB078853
		WB16	<i>Cellulomonas cellasea</i>	98.5	AB078854
		WB17	<i>Cellulomonas cellasea</i>	98.2	AB078855
		W6	<i>Cellulomonas fermentans</i>	98.3	AB078838
		WB18	<i>Cellulomonas fermentans</i>	97.9	AB078856
		Wa	<i>Cellulomonas fimi</i>	97.5	AB078857
		WB2	<i>Cellulomonas gelida</i>	96.1	AB078841
	Propionate producer	W1	<i>Micropruina glycogenica</i>	95.4	AB078835
		Wd	<i>Micropruina glycogenica</i>	95.8	AB078858
		Wf	<i>Micropruina glycogenica</i>	95.7	AB078859
	Others	W7	<i>Microbacterium laevaniformans</i>	99.6	AB078839
<i>Firmicutes</i>	Clostridial group	80Wc	<i>Clostridium populeti</i>	94.1	AB078860
		80Wd	<i>Clostridium josui</i>	97.2	AB078861

Sequence length compared; 1,187-1,392 bases



Table 4. Phylogenetic affiliations of strains isolated from rice stubble with roots in paddy soil

Affiliation	Group	Strain	Closest relative	% Similarity	Accession No.
<i>Bacteroides</i>	<i>Prevotella</i> -like I	KB1	<i>Prevotella pallens</i>	89.8	AB078825
		KB7	<i>Prevotella corporis</i>	92.1	AB078827
		KB9	<i>Prevotella oulorum</i>	92.1	AB078829
		KB12	<i>Prevotella corporis</i>	91.7	AB078832
	<i>Prevotella</i> -like II	KB3	<i>Prevotella oulorum</i>	88.7	AB078826
		KB10	<i>Prevotella bivia</i>	89.4	AB078830
		KB11	<i>Prevotella oulorum</i>	88.6	AB078831
		KB13	<i>Prevotella oulorum</i>	89.3	AB078833
	<i>Cellulomonas</i> -like	K8	<i>Cellulomonas cellasea</i>	98.6	AB078822
		K10	<i>Cellulomonas cellasea</i>	98.5	AB078823
		Ke	<i>Cellulomonas cellasea</i>	98.3	AB078834
		K3	<i>Cellulomonas fermentans</i>	97.9	AB078818
		K6	<i>Cellulomonas fimi</i>	97.5	AB078821
		K12	<i>Cellulomonas fimi</i>	97.7	AB078824
		KB8	<i>Cellulomonas fimi</i>	97.8	AB078828
<i>Actinobacteria</i>	Propionate producer	K2	<i>Micropruina glycogenica</i>	95.9	AB078817
		K5	<i>Microtholunatus phosphovorus</i>	95.1	AB078820
	Others	K4	<i>Propionibacterium acnes</i>	99.6	AB078819
<i>Firmicutes</i>	Clostridial group	KB6	<i>Clostridium butyricum</i>	98.2	AB084626
		80Kb	<i>Clostridium leptum</i>	91.5	AB084627

Sequence length compared; 1,189-1,300 bases

Table 5. Phenotypic characteristics of a representative strain of each bacterial group isolated from plant residue in paddy soil

Characteristic	W5	WB4	W2	W10	Wd	80We	KB7	KB3	Ke	K5
Morphology	Curved rods	Short rods	Irregular rods	Irregular rods	Irregular rods	Rods	Rods	Filaments	Irregular rods	Irregular rods
Cell length (µm)	2.2–3.7	1.3–1.7	1.5–2.0	2.2–3.6	1.8–2.0	2.3–2.4	1.3–2.1	2.2–2.6	1.7–2.6	1.3–1.8
Cell width (µm)	0.6–0.7	0.5–0.6	0.5–0.6	0.7–0.8	0.4–0.5	0.7	0.7–0.8	0.6–0.7	0.4–0.5	0.4–0.5
Gram staining	–	–	+	+	+	+	–	–	+	+
Spore formation	–	–	–	–	–	+	–	–	–	–
Aerobic growth	–	–	+	+	+ <sup>Wn</sup>	–	–	–	+	+ <sup>W</sup>
Oxidase	–	–	–	–	–	–	–	–	–	–
Catalase	–	–	–	–	–	–	–	–	–	–
Nitrate reduction	+	–	+	+	–	+	–	–	+	–
Products from glucose <sup>a</sup>	F, A, E	A, P, S	F, A, L, E	F, A, L, E	A, P, L, S	A, B, E, H <sub>2</sub>	A, M, S	A, P, M, S	F, A, E	A, P, L, S
Utilization of:										
Arabinose	–	+	+	+ <sup>W</sup>	+	+ <sup>W</sup>	+ <sup>W</sup>	+ <sup>W</sup>	+	+
Xylose	–	+ <sup>W</sup>	+	+ <sup>W</sup>	+	+	+	+	–	+
Fructose	+ <sup>W</sup>	+	+	+	+	+	+	+	+ <sup>W</sup>	+
Glucose	+	+	+	+	+	+	+	+	+	+
Cellobiose	+ <sup>W</sup>	+	+ <sup>W</sup>	+	+	+	+	+	+	+
Maltose	–	+	+	+	+	+ <sup>W</sup>	+	+	+	+
Soluble starch	–	+	+	+	–	+	+	+	+	–
Cellulose	–	–	–	–	–	+ <sup>W</sup>	–	–	–	–
Xylane	–	–	–	–	–	+ <sup>W</sup>	+	+	–	–
Phylogenetic group	<i>Betaproteobacteria</i>	<i>Bacteroides</i>	<i>Cellulomonas</i> -like	<i>Cellulomonas</i> -like	Propionate producer	Clostridial	<i>Prevotella</i> -like I	<i>Prevotella</i> -like II	<i>Cellulomonas</i> -like	Propionate producer

<sup>a</sup> Weak<sup>b</sup> F, Formate; A, Acetate; P, Propionate; B, Butyrate; L, Lactate; M, Malate; S, Succinate; E, Ethanol

## Figure legends

Fig. 1. Neighbor-joining tree showing the phylogenetic relationship of the strains isolated from rice straw and stubble with roots in paddy soil aligned with reference strains from the *Betaproteobacteria*, *Bacteroides*, *Actinobacteria*, and *Firmicutes* based on 16S rDNA sequences. Bootstrap values are shown for nodes that had >50% support in a bootstrap analysis of 1,000 replicates. The scale bar represents 2% estimated difference in nucleotide sequence position. As the outgroup, *Escherichia coli* was used.

Fig. 2. Phase-contrast photomicrographs of cells of representative strains isolated from rice plant residue in paddy soil. (A), strain W5 (*Betaproteobacteria* group); (B), strain WB4 (*Bacteroides* group); (C), strain W2 (*Cellulomonas*-like group); (D), strain W10 (*Cellulomonas*-like group); (E), strain Wd (propionate-producing *Actinobacteria* group); (F), strain 80Wc (clostridial group); (G), strain KB7 (*Prevotella*-like group I); (H), strain KB3 (*Prevotella*-like group II); (I), strain Ke (*Cellulomonas*-like group); (J), strain K5 (propionate-producing *Actinobacteria* group). Bar, 10  $\mu\text{m}$  (all panels).

Fig. 1

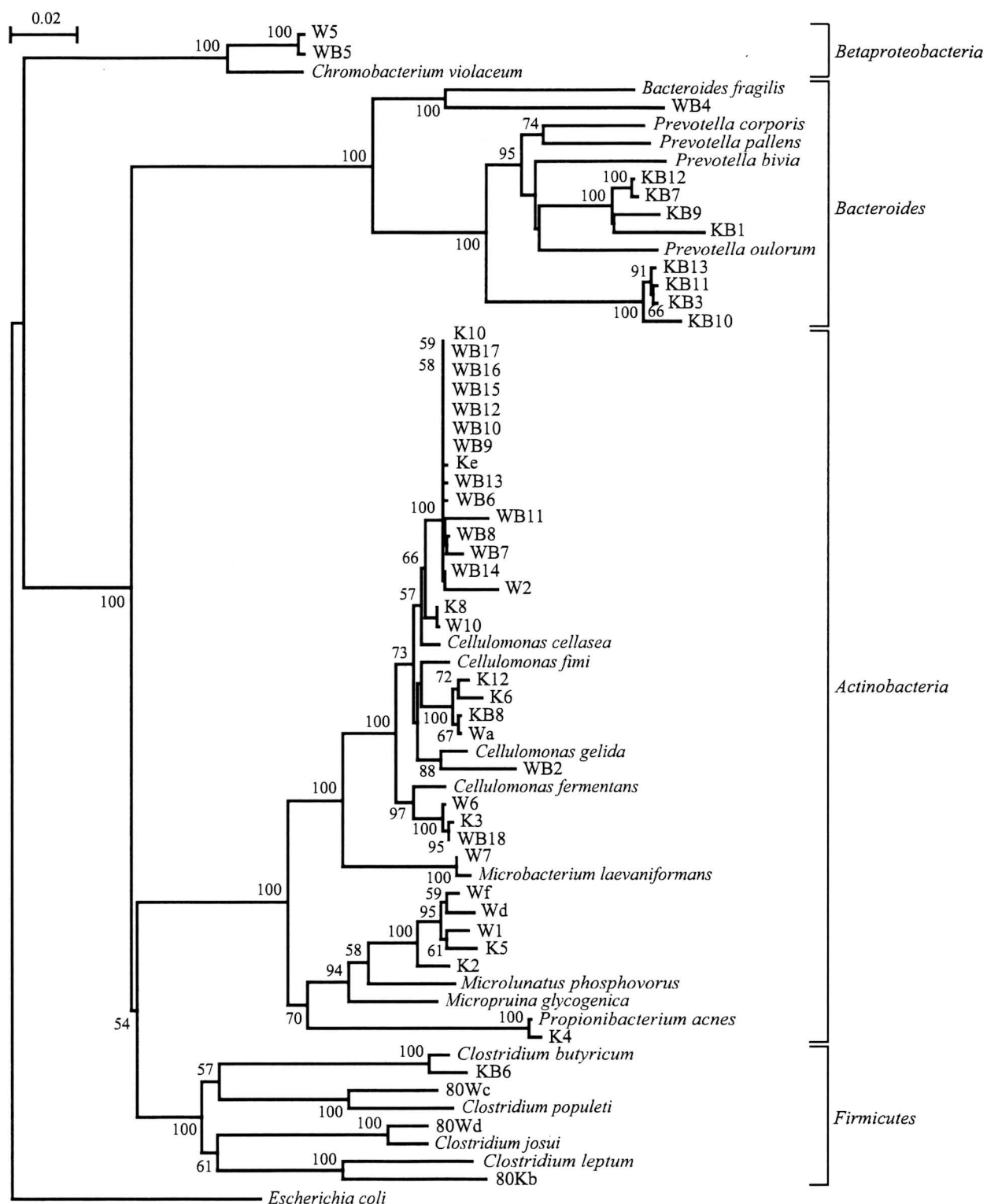


Fig. 2

