Propionicimonas paludicola gen. nov., sp. nov., a new facultatively anaerobic, Gram-positive propionate-producing bacterium isolated from plant residue in irrigated rice field soil

Hiroshi Akasaka[1], Atsuko Ueki[1], Satoshi Hanada[2], Yoichi Kamagata[2], and Katsuji Ueki[1]

[1] Faculty of Agriculture, Yamagata University, Wakaba-machi 1-23, Tsuruoka 997-8555, Japan

[2] Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 6, Higashi 1-1-1, Tsukuba 305-8566, Japan

Author for correspondence: Atsuko Ueki. Tel: +81 235 28 2866. Fax: +81 235 28 2846.

e-mail: uatsuko@tds1.tr.yamagata-u.ac.jp

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The GenBank accession numbers for the 16S rDNA sequences of strains Wd^T and Wf are AB078858 and AB078859, respectively.

SUMMARY

Two propionate-producing strains (Wd^T and Wf) anaerobically isolated from plant residue of irrigated rice field soil in Japan were phenotypically and phylogenetically characterized. The growth rate of strain Wd^T was very slow in the basal medium, while both growth and propionate production were significantly stimulated by the addition of cyanocobalamin. Strain

Wf grew well in the basal medium and produced substantial amounts of fermentation products including propionate. Other phenotypic and phylogenetic characteristics of the two isolates were almost identical. Both were facultatively anaerobic, but much better growth was observed under anaerobic conditions. Cells were Gram-positive, pleomorphic rods with irregular V- or crescent-shaped cell arrangements; non-motile and non-spore-forming. Fermentation products from glucose in the presence of excess amount of cyanocobalamin were acetate, lactate, a small amount of succinate, and CO_2 in addition to propionate. Both oxidase and catalase activities were negative. They possessed *meso*-diaminopimelic acid in their peptidoglycan, and major cellular fatty acids were $C_{13:0}$, anteiso-$C_{15:0}$, and $C_{15:0}$. The isolates contained high genomic G+C contents (68.7 and 67.4 mol%, respectively). Menaquinones, MK-9(H_4) and MK-10(H_4), were dominant respiratory quinones. Phylogenetic analysis based on their 16S rDNA sequences placed both in the *Actinobacteria*, with *Micropruina glycogenica* as their closest relative (sequence similarities of 95.8 and 95.7%, respectively). *Microlunatus phosphovorus* and *Friedmanniella antarctica* were also closely related. As their morphological, physiological and chemotaxonomic characteristics were distinctly different to those of any related species, a new genus and species, *Propionicimonas paludicola*, is proposed for them. The type strain is strain Wd^T (=JCM 11933^T = DSM 15597^T).

INTRODUCTION

Rice serves as the principle food for nearly half of the people on earth, and about 90% of the

total area of rice fields in the world is found in Asia. Rice fields are differentiated into four ecosystems; irrigated, rain-fed, deepwater, and upland fields. The former three soils (about 90% of the global area) (Wassmann *et al.*, 2000b) become anoxic after flooding, and the methanogenic microbial community develops with an active degradation of organic matter (Takai, 1970; Seiler *et al.*, 1984; Glissmann & Conrad, 2000). Thus, the rice field ecosystem is considered one of the major sources of global atmospheric methane (Khalil, 2000). Many studies have looked at the anaerobic microbial communities in anoxic rice field soil (Janssen *et al.*, 1997; Großkopf *et al.*, 1998; Henckel *et al.*, 1999; Hengstmann *et al.*, 1999), and new anaerobic microorganism have been isolated (Rajagopal *et al.*, 1988; Asakawa *et al.*, 1995; Chin *et al.*, 1998; Chin *et al.*, 1999; Rosencrantz *et al.*, 1999; Wind *et al.*, 1999; Satoh *et al.*, 2002).

Japan is located in the rice-producing district of Asia, and rice cultivation using irrigated fields has been practiced for a long period. Rice straw and remaining plant residue such as rice stubble and roots are plowed into soil at the beginning of the growing season as organic fertilizers. Such additions often results in an increase in methane emission rates from the fields (Yagi & Minami, 1990; Watanabe *et al.*, 1995; Wassman *et al.*, 2000a). In a previous study, we reported enumeration and isolation of anaerobic fermentative bacteria from rice plant residue plowed into soil of a Japanese rice field (Akasaka *et al.*, 2003). Of the 47 bacterial isolates analyzed with the 16S rDNA sequences, five strains in the *Actinobacteria* commonly produced

propionate from glucose. This group was phylogenetically distinct and was tentatively named the "propionate-producing *Actinobacteria* group". We have investigated phenotypic characteristics of representative strains, and from the results obtained we propose here a new genus and species name, *Propionicimonas paludicola*, for these isolates.

METHODS

Bacterial strains.

Strains W1, Wd^T (= JCM 11933^T = DSM 15597^T), Wf (= JCM 11934 = DSM 15598), K2, and K5 in the "propionate-producing *Actinobacteria* group" were previously isolated from plant residue samples collected from the rice straw (RS) plot in the Shonai Branch of the Yamagata Agricultural Experimental Station (Fujishima-machi, Yamagata, Japan) during the rice growing season (Akasaka *et al.*, 2003). Rice straw had been applied annually to this plot for more than 20 years. Cultivation practices for rice plants and characteristics of the soil were described previously (Ueki *et al.*, 1999; Kaku *et al.*, 2000; Ueki *et al.*, 2000; Hattori *et al.*, 2001; Hattori *et al.*, 2002). Strains were isolated by selecting individual colonies after counting anaerobic bacteria on plant residue collected from the soil. The methods used were described previously (Hungate, 1966; Holdeman *et al.*, 1977; Akasaka *et al.*, 2003). Strains W1, Wd^T, and Wf were isolated from rice straw samples, while strains K2 and K5 were derived from rice stubble and roots. Strains W1, K2, and K5 were isolated in May during the flooding period of the field, and the others were isolated in August during the intermittent

irrigation period (Akasaka *et al.*, 2003).

Culture conditions.

The strains were cultivated anaerobically at 30°C using PY medium as a basal medium with oxygen-free N_2 95%-CO_2 5% mixed gas as headspace as described previously (Akasaka *et al.*, 2003). PY medium supplemented (l^{-1}) with 0.25 g each of glucose, cellobiose, maltose, and soluble starch, as well as 50 ml plant residue extract (RE) and 15 g agar (Difco Laboratories), was designated PY4SR agar and used for the maintenance of the strains in agar slants. Plant residue collected from the RS plot during the flooding period was autoclaved (120°C for 30 min) with a fivefold amount (wet weight basis) of deionized H_2O, and the supernatant obtained after centrifugation was used as RE for the provision of growth factors. PY liquid medium supplemented with glucose at 10 g l^{-1} (PYG medium) was usually used for cultivation of the cells. For the PY liquid medium, cyanocobalamin (cobalamin) (vitamin B_{12}, Kanto Chemical) (50 μg l^{-1}) was added as a growth factor as described below, unless otherwise stated. The media were adjusted to pH 7.3 with 1 M NaOH. For estimation of optimal growth conditions, strains were cultivated under different conditions of temperature (in the range of 10- 50°C), pH (in the range of pH 4.6-7.5 of PYG medium) and NaCl concentrations (in the range of 0-5.0% in PYG medium). Growth in the liquid medium was monitored by optical density changes at 660 nm.

Morphological characteristics.

Morphology of cells was observed by Gram-staining, phase-contrast microscopy, and transmission electron microscopy (TEM). For TEM a centrifuged cell pellet was fixed with 5% (v/v) glutaraldehyde and 1% (v/v) osmium tetraoxide. Ultrathin sections of the sample embedded in epoxy resin (Kushida, 1980) were prepared with a Reichert ultramicrotome. Samples were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7000 transmission electron microscope.

Physiological and biochemical characterization.

Growth of strains under aerobic conditions was examined by plate culture on nutrient agar (Nissui Pharmacy) and PY4SR agar media. Spore formation was assessed after Gram-staining as well as under phase-contrast microscopy, and by subsequent growth in PYG medium of cells exposed to 80°C for 10 min. Oxidase, catalase, and nitrate-reducing ability were determined according to the methods as described previously (Satoh et al, 2002; Akasaka et al, 2003). Utilization of carbon sources was tested in the PY liquid medium with each substrate added at 10 g l^{-1} (for monosaccharides, disaccharides, trisaccharides, polysaccharides, or sugar alcohols) or 30 mM (for alcohols and organic acids). Fermentation products were analyzed as descried previously (Ueki *et al.*, 1986; Akasaka *et al.*, 2003). Glucose concentrations in the media were measured with a D-Glucose kit (Boehringer Mannheim GmbH) according to the manufacturer's protocol. All phenotypic characterizations including the following

chemotaxonomic analyses were carried out in duplicate.

Quinone, fatty acid and cell wall analyses.

Quinones were extracted from freeze-dried cells with chloroform/methanol (2:1, v/v) and n-hexane. Extracts were purified by Sep-Pak Plus (Waters) and analyzed by reverse-phase HPLC for identification (Tamaoka *et al.*, 1983). Whole-cell fatty acids were converted to methyl esters by treatment with anhydrous methanolic HCl (Ueki & Suto, 1979; Komagata & Suzuki, 1987). Methyl esters were extracted with n-hexane and analyzed using a GC/MS (Hitachi M7200A GC/3DQMS) equipped with a DB-5ms capillary column coated with (5%-phenyl)-methylpolysiloxane at a thickness of 250 nm (Hanada *et al.*, 2002). The presence of diaminopimelic acid (DAP) isomers in the cell wall peptidoglycan was determined by TLC (Merck; no. 5716) after hydrolysis with 6 M HCl at 100°C for 18 h (Komagata & Suzuki, 1987).

DNA base composition.

Genomic DNA was extracted according to the method described previously (Kamagata & Mikami, 1991). Extracted DNA was digested with P1 nuclease using a Yamasa GC kit (Yamasa Shoyu), and its G + C content was measured by HPLC (Shimadzu model LC-6A system) equipped with a CLC-ODS column (6 by 150 mm) (Shimadzu).

16S rDNA sequence and phylogenetic analysis.

The 16S rDNA of strains was extracted according to the method described previously (Akasaka *et al.*, 2003) and amplified by PCR. PCR-amplified 16S rDNA was sequenced using the Thermo sequenase cycle sequencing kit (Amersham Pharmacia Biotech) and a DNA sequencer model 4000L (LI-COR). Multiple alignments of sequences of isolates with the reference sequences in the GenBank database were performed with the BLAST program (Altschul *et al.*, 1997). A phylogenetic tree with the neighbor-joining method (Saitou & Nei, 1987) was constructed using the CLUSTAL W program (Thompson *et al.*, 1994). All gaps and unidentified base positions in the alignments were excluded before assemblages.

RESULTS AND DISSCUSION

Cobalamin requirement of the isolates.

The five isolates have 16S rDNA similarities of 98.0-99.7% each other, and all are facultatively anaerobic, Gram-positive, irregular rods with a similar morphology (Akasaka *et al.*, 2003). However, the five isolates could be divided into two groups according to their growth in PYG medium. Four isolates (W1, Wd^T, K2, and K5) showed extremely slow growth rates without addition of any growth factors, while strain Wf grew well. For slow-growing isolates, 16S rDNA similarities among three strains (W1, Wd^T, and K5) were in the range of 99.4-99.7%, while those with strain K2 were lower (98.0-98.3%).

By screening possible growth factors, addition of cobalamin to PYG medium markedly stimulated growth of the four slow-growing strains. Table 1 shows the effects of cobalamin addition on growth (final OD660) and fermentation end products. In PYG medium without added cobalamin, all isolates except strain Wf produced small amounts of products, with lactate as the predominant one. In the presence of added cobalamin, production of propionate was stimulated together with acetate, while lactate changed to a minor product. Although strain Wf produced large amounts of fatty acids in the PYG medium with no growth factor addition, cobalamin also stimulated its growth and propionate production again with a concomitant suppression of lactate production. All isolates produced CO_2 as well as fatty acids, but hydrogen production was not observed.

To further investigate effects of cobalamin on slow-growing isolates, strain Wd^T was cultivated in the presence of different amounts of cobalamin in PYG medium, and time courses for growth and end products were examined (data not shown). Without added cobalamin, strain Wd^T grew very slowly, showing an arithmetic increase in its optical density. With addition of 1 μg cobalamin l^{-1}, its growth was markedly improved, and lactate was produced as the most dominant fatty acids from the onset of fermentation. With 20 μg cobalamin l^{-1}, propionate was rapidly accumulated to a much higher concentration than any other products, while lactate production was significantly suppressed than that in the presence of 1 μg cobalamin l^{-1}. Other three slow-growing strains (W1, K2, and K5) showed similar

changes in time course of growth depending on cobalamin concentrations.

Strains Wd^T and Wf were selected and characterized in more detail. In the later experiments, both isolates were cultured in media containing an excess amount of cobalamin (50 $\mu g\ l^{-1}$).

Colony and cell morphologies.

Strains Wd^T and Wf grew anaerobically on PY4SR agar and produced white colonies of 2-3 mm in diameter after 2-3 d of incubation. Cells were Gram-positive, pleomorphic, often slightly curved rods, 0.4-0.5 μm in diameter and 1.4-2.2 μm in length. Irregular V- or crescent-shaped cell arrangements were frequently observed (Fig. 1a, c). Cells were non-motile under phase-contrast. TEM showed that cells had a cell wall structure of typical Gram-positive bacteria (Fig. 1b, d). Electron translucent regions in the cells revealed the presence of intracellular storage compounds. Spore formation was not observed, and cells treated at 80°C for 10 min did not grow.

Physiological and biochemical characteristics.

Strains Wd^T and Wf showed very similar physiological and biochemical characteristics. Both grew weakly under air on both PY4SR and nutrient agar, but much better growth was obtained under anaerobic conditions. Both utilized arabinose, xylose, fructose, galactose, glucose, mannose, cellobiose, maltose, saccharose, trehalose, glycerol, and mannitol as carbon sources,

whereas utilization of ribose and lactose was poor. Both grew on pyruvate and lactate, and very weak growth was observed with malate, fumarate, and succinate. Neither utilized fucose, rhamnose, sorbose, melibiose, melezitose, raffinose, cellulose, glycogen, soluble starch, xylan, adonitol, dulcitol, erythritol, inositol, sorbitol, ethanol, methanol, or propanol as growth substrates. Other physiological characteristics of the two isolates are shown in Table 2.

Chemotaxonomic characteristics.

Strains Wd^T and Wf also had similar chemotaxonomic characteristics. The major cellular fatty acids in both were $C_{13:0}$ (11.8% for strain Wd^T and 10.5% for strain Wf), anteiso-$C_{15:0}$ (31.0 and 39.6%, respectively) and $C_{15:0}$ (40.6 and 34.1%, respectively). Branched-$C_{14:0}$ (5.1 and 5.5%), $C_{14:0}$ (1.5 and 2.8%), $C_{16:0}$ (1.7 and 1.4%), and $C_{17:0}$ (4.1 and 1.5%) were also detected as minor components. Major respiratory quinones of both were MK-9(H_4) and MK-10(H_4) with peak area ratios of 59:41 for strain Wd^T and 77:23 for strain Wf. Other chemotaxonomic features are also given in Table 2.

Phylogenetic analysis based on the 16S rDNA sequences.

A phylogenetic tree based on the 16S rDNA sequences was constructed (Fig. 2). The similarity between the 16S rDNA of strains Wd^T and Wf was 99.1%, and both formed a cluster close to the genera *Micropruina* (Shintani *et al.*, 2000), *Microlunatus* (Nakamura *et al.*, 1995) and *Friedmanniella* (Schumann *et al.*, 1997) in the *Actinobacteria*. The closest relative to both was

Micropruina glycogenica (Shintani *et al.*, 2000), with sequence similarities of 95.8% and 95.7 %, respectively. The closest relative to strain Wd^T found in the database was an environmental clone SJA-181 (Fig. 2), which was derived from an anaerobic microbial consortium in a trichlorobenzene-transforming bioreactor (von Wintzingerode *et al.*, 1999) with a 16S rDNA similarity of 98.3%.

The five strains used in this study were isolated from different plant residues at different sampling times, and thus were considered one of ubiquitous anaerobic bacterial group occurring on plant residue in rice field soil. Their population density was estimated to be at least at the order of 10^8-10^9 CFU (g of dry weight of plant residue)$^{-1}$ (Akasaka *et al.*, 2003). Propionate is an important intermediate in methanogenic decomposition of organic matter in anoxic rice field soils (Dolfing 1988; Glissmann & Conrad, 2000; Chin *et al.*, 2001; Chin & Janssen, 2002). The striking effects of cobalamin on growth and fermentation of the isolates suggest that survival of the group on plant residue at a rather high population depends on the availability of cobalamin from the environment. A similar requirement for cobalamin in a ruminal propionate-producing bacterium, *Prevotella ruminicola*, has been reported (Strobel, 1992).

Many propionate-producing bacteria produce propionate through conversion of succinyl-CoA to methylmalonyl-CoA, which is catalyzed by a vitamin B_{12}-dependent methylmalonyl CoA

isomerase (Roth *et al.*, 1996). Stimulation of propionate production by added cobalamin suggests the presence of this pathway in the isolates described here. Many other anaerobes including methanogens and acetogens synthesize cobalamin endogenously for their own use, and several reactions including reductive dehalogenation of chlorinated compounds depend on cobalamin or vitamin B_{12} (Krone *et al.*, 1989; Stupperich *et al.*, 1990; White & Zhou, 1993; Roth *et al.*, 1996). Thus, the cobalamin synthesized by various microbial groups may be released into the environment and hence support there the growth of the cobalamin-requiring bacteria.

The phenotypic and phylogenetic characteristics of strains Wd^T and Wf were almost the same except for a requirement for cobalamin for their optimal growth. *Micropruina glycogenica* was the most closely related known species to both isolates based on the 16S rDNA sequences as described above, followed by *Microlunatus phosphovorus* (Nakamura *et al.*, 1995) (similarity of 94.7-95.8%), and *Friedmanniella antarctica* (Schumann *et al.*, 1997) (similarity of 93.1%), all in the family *Propionibacteriaceae* within the *Actinobacteria* (Garrity & Holt, 2001) (Fig. 2). These data agree with the base compositions of their genomic DNA which indicate that they both belong to the high G+C Gram-positive bacteria. However, many differences exist between the characteristics of these isolates and those of other closely related species (Table 2).

Their cellular morphologies are irregular rods, while the other relatives have spherical cells. The isolates are facultatively anaerobic and catalase negative, and grow well under anoxic conditions, while the other three are strictly aerobic and catalase positive. Both *Micropruina glycogenica* and *Microlunatus phosphovorus* have oxidase and nitrate-reducing activities, but these isolates do not. Furthermore, their cellular fatty acid composition profiles are different to their close relatives. Major fatty acids of these isolates are $C_{13:0}$, anteiso-$C_{15:0}$, and $C_{15:0}$, while those of the other three are iso-$C_{14:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$ or $C_{16:0}$. $C_{13:0}$, and $C_{15:0}$ fatty acids are absent from or only minor components in other related species. Furthermore, most predominant fatty acids in their relatives except for anteiso-$C_{15:0}$ are absent from or only minor components in the isolates described here. In addition to MK-9(H_4), both isolates contain MK-10(H_4) as a predominant menaquinone, while the other three species do not (Nakamura *et al.*, 1995; Schumann *et al.*, 1997; Maszenan *et al.*, 1999; Shintani *et al.*, 2000). Among their close relatives, only *Micropruina glycogenica* has *meso*-DAP in the peptidoglycan of the cell wall (Shintani *et al.*, 2000).

The isolates produce propionate in the presence of excess cobalamin, and their fermentation patterns resemble those of *Propionibacterium* spp. (Cummins & Johnson, 1986) and *Propioniferax* spp. (Yokota *et al.*, 1994; Pitcher & Collins, 1991) in the family *Propionibacteriaceae* (Garrity & Holt, 2001). Even though, they are only distantly related to them (*Propionibacterium propionicus* and *Propioniferax innocua*) based on 16S rDNA

sequences data (similarities of 91.5-92.0% and 90.0%, respectively) (Fig. 2). Furthermore, both *Propionibacterium propionicus* and *Propioniferax innocua* contain $_{LL}$-DAP in the peptidoglycan of cell wall and MK-9(H_4) as the sole menaquinone (Maszenan *et al.*, 1999). Predominant cellular fatty acids in *Propioniferax innocua* are also iso-$C_{15:0}$ and anteiso-$C_{15:0}$ (Schumann *et al.*, 1997).

On the basis of both the phylogenetic and phenotypic characteristics described above, we conclude that Wd^T and Wf should be affiliated with the same species and assigned to a new genus, *Propionicimonas* gen. nov., with *Propionicimonas paludicola* sp. nov. as a type species.

Description of *Propionicimonas* gen. nov.

Propionicimonas (Pro. pi. on. i. ci. mo´ nas. N.L. n. *acidum propionicum* propionic acid; Gr. n. *monas* a unit, monad; N.L. fem. n. *Propionicimonas*, propionic acid-producing monad). Cells are Gram-positive irregular rods. Facultatively anaerobic chemoorganotroph. Non-motile. and non-spore forming. Mesophilic. Oxidase, catalase and nitrate reduction negative. Propionate is produced anaerobically from glucose. Cell wall peptidoglycan contains *meso*-DAP, and major cellular fatty acids are $C_{13:0}$, anteiso-$C_{15:0}$, and $C_{15:0}$, MK-9(H_4) and MK-10(H_4) as the major quinones. The G+C content of the genomic DNA is 68.7 mol%. On the basis of the 16S rDNA sequences, the bacterium belongs to the *Actinobacteria* phylum. The genera *Micropruina, Microlunatus* and *Friedmanniella* are most closely related to this genus. Type species is

Propionicimonas paludicola.

Description of *Propionicimonas paludicola* sp. nov.

Propionicimonas paludicola (pa. lu. di´co. la. L. n. *palus -udis* a swamp, marsh; L. suff. *-cola* derived from incola, inhabitant, dweller; N.L. masc. n. *paludicola*, an inhabitant of swamps). Gram-positive irregular, often slightly curved rods, 0.4-0.5 μm in diameter and 1.8-2.0 μm in length, and frequently arranged in irregular V- or crescent-shapes. Optimal growth occurs at 35°C and at pH 6.5. The species grows in the presence of NaCl up to 2.0% (w/v). Cells produce acetate, propionate, lactate, succinate, and CO_2 anaerobically from glucose. Growth and production of propionate are both stimulated by cyanocobalamin addition to the medium. Cells grow on arabinose, xylose, fructose, galactose, glucose, mannose, cellobiose, maltose, saccharose, trehalose, glycerol, mannitol, pyruvate, and lactate, but growth is scarce on ribose, lactose, malate, fumarate, and succinate. Fucose, rhamnose, sorbose, melibiose, melezitose, raffinose, cellulose, glycogen, soluble starch, xylan, adonitol, dulcitol, erythritol, inositol, sorbitol, ethanol, methanol, or propanol are not utilized. The chemotaxonomic characteristics of the species are the same as those described for the genus. The type strain is Wd^T, which has been deposited in the Japan Collection of Microorganisms (RIKEN) as JCM 11933^T (=DSM 15597^T).

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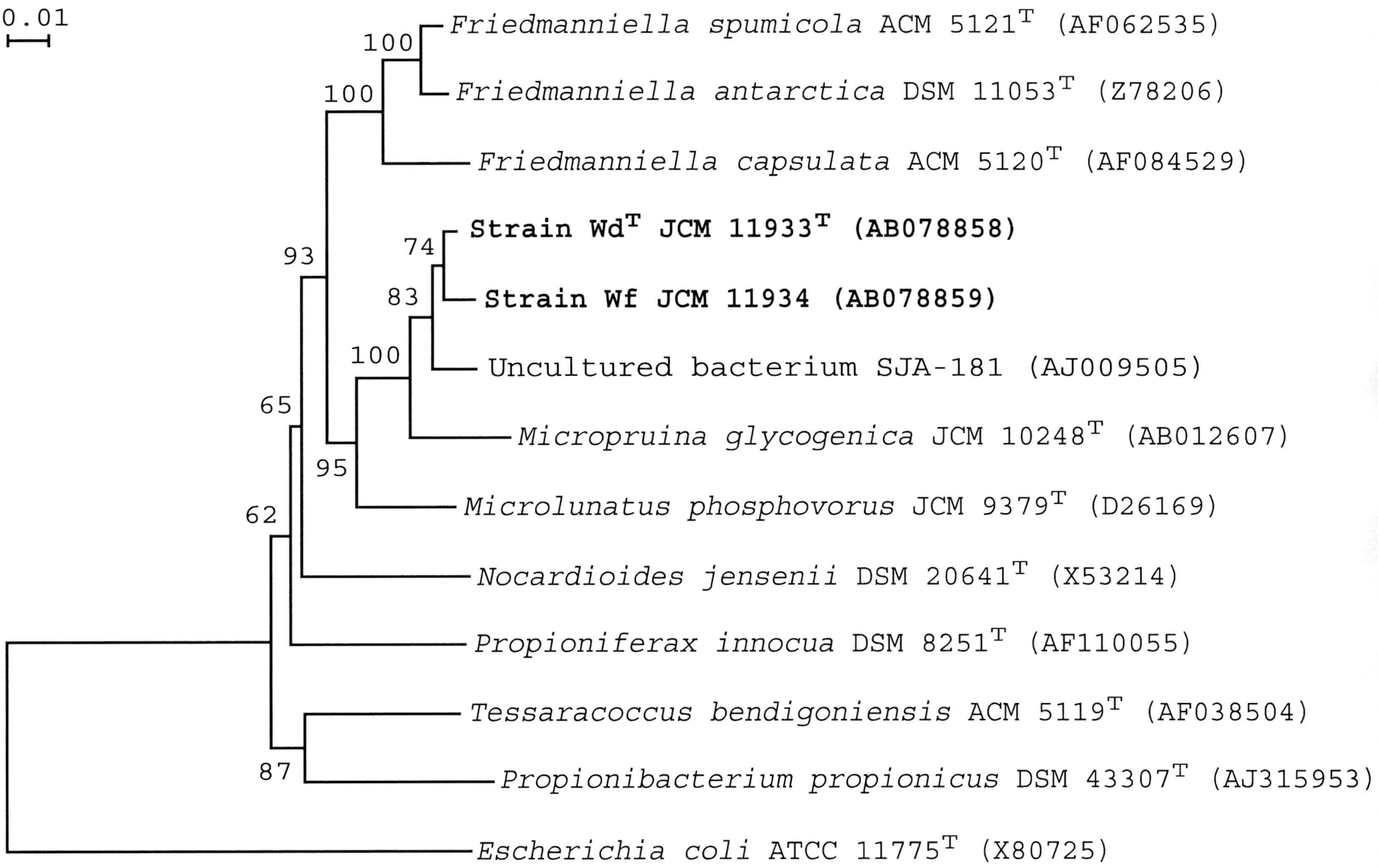
Figure legends

Fig. 1. Neighbor-joining tree showing the phylogenetic relationship of strains Wd^T, Wf and related species in the *Actinobacteria* based on 16S rDNA sequences. Bootstrap values (%) are shown for nodes that had >50% support in a bootstrap analysis of 1000 replicates. The scale bar represents 1% estimated difference in nucleotide sequence position. The sequence of *Escherichia coli*, which belongs to the *Gammaproteobacteria* (Garrity & Holt, 2001), was used as the outgroup.

Supplementary figures a-d. Cell morphologies of strains Wd^T (a, b) and Wf (c, d). Phase-contrast photomicrographs of cells (a, c) grown anaerobically on agar slants of PY4SR. Bars, 10 μm. Transmission electron photomicrographs of cells (b, d) grown in PYG liquid medium in the presence of 50 μg cobalamin l^{-1} under anaerobic condition. Bars, 0.5 μm.

Fig. 1


0.01
100
100
93
74
83
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95
62
87
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Friedmanniella antarctica DSM 11053T (Z78206)
Friedmanniella capsulata ACM 5120T (AF084529)
Strain WdT JCM 11933T (AB078858)
Strain Wf JCM 11934 (AB078859)
Uncultured bacterium SJA-181 (AJ009505)
Micropruina glycogenica JCM 10248T (AB012607)
Microlunatus phosphovorus JCM 9379T (D26169)
Nocardioides jensenii DSM 20641T (X53214)
Propioniferax innocua DSM 8251T (AF110055)
Tessaracoccus bendigoniensis ACM 5119T (AF038504)
Propionibacterium propionicus DSM 43307T (AJ315953)
Escherichia coli ATCC 11775T (X80725)

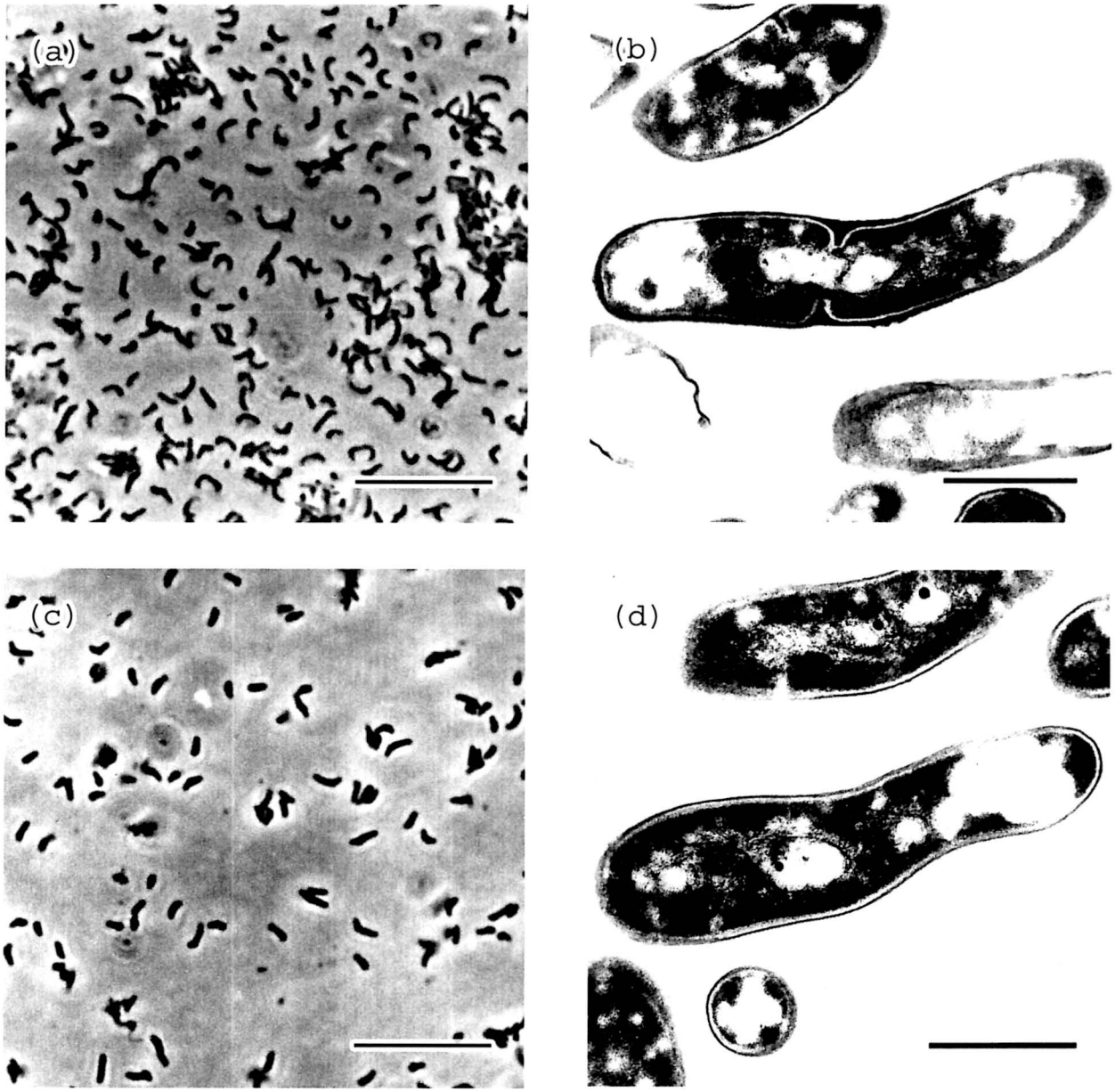
(a)
(b)
(c)
(d)

Table 1. Effects of addition of cyanocobalamin on growth and fermentation products of isolates in the propionate-producing group

All values were determined after 5 d incubation.
–, without cobalamin added; +, with cobalamin added; nd, not detected.

Strain	Cyanocobalamin*	OD 660	pH	Fatty acids produced (mmol/l)			
				Acetate	Propionate	Lactate	Succinate
W1	–	0.28	6.22	0.54	0.80	2.29	0.11
	+	2.21	4.60	8.12	17.5	0.49	nd
Wd	–	0.71	5.70	2.46	0.59	4.26	2.45
	+	2.04	4.42	8.06	17.7	3.21	0.89
K2	–	0.53	5.67	0.92	0.74	4.27	0.32
	+	2.11	4.52	9.47	19.0	nd	nd
K5	–	0.29	6.26	1.29	0.64	3.95	0.36
	+	1.63	4.48	6.49	20.5	1.69	0.38
Wf	–	1.87	4.35	8.12	12.3	15.2	0.53
	+	2.40	4.34	13.8	24.7	6.01	0.27

*Amounts of cyanocobalamin added: strain Wd, K5 and Wf, 10 $\mu g\ l^{-1}$; W1 and K2, 50 $\mu g\ l^{-1}$.

Table 2. Phenotypic characteristics of strain Wd^T, Wf and related species in the *Actinobacteria*

All organisms are Gram-positive, non-motile, and non-spore-forming.
Data were taken from the following references: *Micropruina glycogenica*, (Shintani *et al*., 2000); *Microlunatus phosphovorus*, (Nakamura *et al*., 1995); *Friedmanniella antarctica*, (Schumann *et al*., 1997).
+, Positive; –, negative; ND, no data available.

Characteristic	Wd^T	Wf	*Micropruina glycogenica*	*Microlunatus phosphovorus*	*Friedmanniella antarctica*
Habitat	Plant residue in paddy soil	Plant residue in paddy soil	Activated sludge reactor	Activated sludge reactor	Antarctic sandstone
Cell shape	Irregular rods	Irregular rods	Cocci	Cocci	Cocci
Cell size (μm)	0.4–0.5×1.8–2.0	0.4–0.5×1.4–2.2	0.5–2.2	0.8–2.0	0.5–2.2
Color of colony	White	White	White	Cream	Orange
Optimum growth temperature (°C)	35	35	30	25–30	22
Growth temperature range (°C)	10–40	10–40	20–30	5–35	9–25
Optimum growth pH	6.5	6.5	7.0	7.0	6.0–7.2
Growth pH range	4.5–7.5	4.5–7.5	6.0–8.0	5.0–9.0	5.1–8.7
Growth NaCl concn. range (%)	0–2	0–2	0–3	0–6	0–2
O_2 requirement	Facultative anaerobe	Facultative anaerobe	Aerobe	Aerobe	Aerobe
Cobalamin requirement	+	–	ND	ND	ND
Oxidase	–	–	+	+	–
Catalase	–	–	+	+	+
Nitrate reduction	–	–	+	+	–
Acid production from glucose	+	+	+	+	–
DNA G+C content (%)	68.7	67.4	70.5	67.9	73.0
Major quinone	MK-9(H_4), MK-10(H_4)	MK-9(H_4), MK-10(H_4)	MK-9(H_4)	MK-9(H_4)	MK-9(H_4)
Major cellular fatty acids	$C_{13:0}$, anteiso-$C_{15:0}$ $C_{15:0}$	$C_{13:0}$, anteiso-$C_{15:0}$ $C_{15:0}$	iso-$C_{14:0}$, anteiso-$C_{15:0}$ iso-$C_{16:0}$, $C_{16:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$ iso-$C_{16:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$
Peptidoglycan	*meso*-DAP	*meso*-DAP	*meso*-DAP	LL-DAP	LL-DAP