COVER SHEET

Paludibacter propionicigenes gen. nov., sp. nov., a novel strictly-anaerobic, Gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan

Atsuko Ueki[1], Hiroshi Akasaka[1,2], Daisuke Suzuki[1], and Katsuji Ueki[1]

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

[2] Present address: Creative Research Initiative 'Sousei' (CRIS), Hokkaido University, Kita 21, Nishi 10, Kita-ku, Sapporo, 001-0021, Japan

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

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

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SUMMARY

A strictly anaerobic, propionate-producing strain (WB4^T) isolated from rice plant residue in anoxic rice-field soil in Japan was characterized phenotypically and phylogenetically. Cells were Gram-negative, non-motile, non-spore-forming, short rods. The strain utilized various sugars and produced propionate and acetate as major fermentation products with a small

amount of succinate. The optimum growth temperature was 30ºC. Oxidase, catalase and nitrate-reducing activities were negative. The major cellular fatty acids were anteiso-$C_{15:0}$, $C_{15:0}$ and anteiso-3-OH $C_{17:0}$. Menaquinone MK-8(H_4) was the major respiratory quinone. The genomic DNA G + C content was 39.3 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence placed the strain in the *Bacteroidetes*. The closest relative to strain WB4^T was an environmental clone from water contaminated with equine manure (sequence similarity of 99.7%) and the strain formed a distinct cluster with other environmental clones mainly from freshwater sediments. The closest recognized species were members of the genus *Dysgonomonas*, with 16S rRNA gene sequence similarities of 90.9-89.8%. *Bacteroides merdae* was the next closest recognized species (similarity of 88.7% to the type strain). Given that the ecological, physiological and chemotaxonomic characteristics of strain WB4^T were different from those of any related species, a new genus and species *Paludibacter propionicigenes* gen. nov., sp. nov. is proposed to accommodate it. The type strain is WB4^T (= JCM 13257^T = DSM 17365^T).

MAIN TEXT

Rice is extensively cultivated using irrigated field systems in Japan. During the flooding period of the rice fields, the methanogenic microbial community consisting of various fermentative, anaerobically respiring and methanogenic microbes develops under anoxic conditions in soil, and methane, a major greenhouse gas, is emitted into the atmosphere (Takai,

1970; Seiler *et al*., 1984; Khalil, 2000; Wassmann *et al*., 2000a,b). Many studies have investigated the microbial communities in flooded rice-field soil (Janssen *et al*., 1997; Großkopf *et al*., 1998; Henckel *et al*., 1999; Hengstmann *et al*., 1999). In anoxic rice-field soil, diverse fermentative bacterial groups play a key role in decomposition of organic matter such as rice plant residue ploughed into the soil and exudate from roots of rice plants, thus producing substrates such as acetate and H_2 for methanogenesis (Cicerone & Oremland, 1988; Boon, 2000; Glissmann & Conrad, 2000; Weber *et al*., 2001).

We have isolated various fermentative anaerobes from plant residue (rice straw and rice stubble) and living rice roots in irrigated rice-field soil in the course of investigations on microbes in this environment (Satoh *et al*. 2002; Akasaka *et al*., 2003a,b; Akasaka *et al*., 2004). In this study, we have characterized one of the isolates (strain WB4^T) from plant residue samples (Akasaka *et al*., 2003a; Akasaka *et al*., 2004), and found it to be phylogenetically distant from any related recognized species. The isolate was a propionate-producing, strictly anaerobic bacterium with cells that are Gram-negative, non-motile rods. Physiological and chemotaxonomic characteristics supported the proposal of a novel genus and species in the phylum *Bacteroidetes* to accommodate the strain.

Strain WB4^T was isolated from a rice plant residue (rice straw) sample collected from the irrigated rice-field soil in the Shonai Branch of the Yamagata Agricultural Experimental

Station (Fujishima-machi, Yamagata, Japan) during the flooding period of the field (Akasaka *et al*., 2003a, 2004). Cultivation practices for rice plants and other conditions of the fields were described in Ueki *et al*. (2000). The strain was isolated by using the anaerobic roll tube method for enumeration of anaerobic fermentative bacteria by the colony-counting method (Hungate, 1966; Holdeman *et al*., 1977; Akasaka *et al*., 2003a; Akasaka *et al*., 2004).

The strain was cultivated anaerobically at 30ºC unless otherwise stated by using peptone/yeast extract (PY) medium as basal medium with oxygen-free, 95% N_2/ 5% CO_2 mixed gas as the headspace, as described by Akasaka *et al*. (2003a). PY medium supplemented with (l^{-1}) 0.25 g each of glucose, cellobiose, maltose and soluble starch as well as 15 g agar (Difco) was designated PY4S agar and used for maintenance of the strain in agar slants. PY liquid medium supplemented with 10 g glucose l^{-1} (PYG medium) was usually used for cultivation of the cells. Growth in liquid medium was monitored by changes in OD_{660}.

Growth of the strain under aerobic conditions was examined by plate culture on nutrient agar (Nissui Pharmacy) and PY4S agar modified to exclude Na_2CO_3, cysteine-HCl-H_2O and resazurine-Na. Spore formation was assessed by observation of cells after Gram-staining and by growth in PYG medium of cells exposed to 80ºC for 10 min. Oxidase, catalase and nitrate-reducing activities were determined according to the methods described by Satoh *et al*. (2002) and Akasaka *et al*. (2003a,b). Utilization of carbon sources was tested in PY liquid

medium with each substrate added at 10 g^{-1} (for sugars and sugar alcohols) or 30 mM (for alcohols and organic acids). Bile sensitivity was determined by the addition of bile salts (Oxioid, UK) (0.1-0.5%, w/v) to PYG medium (Lawson *et al*., 2002). Fermentation products were analyzed by GC or HPLC as described previously (Ueki *et al*., 1986; Akasaka *et al*., 2003a). Other characterization was performed according to the methods as described by Holdeman *et al*. (1977) and Akasaka *et al*. (2003a).

Whole-cell fatty acids (CFAs) were converted to methyl esters by saponification, methylation and extraction according to the method of Miller (1982). Methyl esters of CFAs were analyzed by GC (Hp6890, Hewlett-Packard or G-3000, Hitachi) equipped with a HP Ultra 2 column. CFAs were identified by equivalent chain-length (ECL) (Miyagawa *et al*., 1979; Ueki & Suto, 1979) according to the protocol of NCIMB Japan based on the MIDI microbial identification system (Microbial ID) as described by Moore *et al*. (1994). Microbial identification system of the TSBA40 was used to confirm identification. Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analyzed by using a mass spectrometer (JMS-SX102A, JEOL). Genomic DNA was extracted according to the method described by Kamagata & Mikami (1991). Extracted DNA was digested with P1 nuclease by using YAMASA GC kit (Yamasa shoyu) and its G + C content was measured by HPLC (HITACHI L-7400) equipped with a µBondapack C18 column (3.9 x 300 mm; Waters).

Genomic DNA was extracted and the 16S rRNA gene was amplified by PCR according to the method described by Akasaka *et al*. (2003a,b). The PCR-amplified 16S rRNA gene was sequenced by using a Thermo Sequenase Primer Cycle Sequencing kit (Amersham Biosciences) and a DNA sequencer (4000L; Li-COR). Multiple alignments of the sequence with reference sequences in GenBank were performed with the BLAST program (Altschul *et al*., 1997). A phylogenetic tree was constructed with the neighbor-joining method (Saitou & Nei, 1987) by using the CLUSTAL W program (Thompson *et al*., 1994). All gaps and unidentified base position in the alignments were excluded before sequence assembly.

Cells of strain WB4^T were Gram-negative, short rods, 0.5-0.6 μm in diameter and 1.3-1.7 μm in length. Ends of cells were usually round to slightly tapered (Fig. 1). Longer cells were occasionally formed both singly and in chains of the short cells. Spherical cells sometimes occurred after storage of slant cultures for 1-2 months at 4ºC. Cells were non-motile under phase-contrast microscopy. The strain could not grow in air on both PY4S and nutrient agar. Colonies on PY4S agar were greyish white, translucent and circular with a smooth surface and were 1-1.5 mm diameter after 48 h anaerobic incubation. When freshly cultivated cells were used as an inoculum, the strain grew rapidly in PYG liquid medium at a specific growth rate (μ) of 0.343 h^{-1} at 30ºC; however, inoculation from older cultures sometimes significantly delayed growth. Spore formation was not observed and cells treated at 80ºC for 10 min. did not grow.

Both catalase and oxidase were negative and the strain did not reduce nitrate. The strain utilized arabinose, xylose, cellobiose, fructose, galactose, glucose, mannose, maltose, melibiose, glycogen and soluble starch as growth substrates and produced acids. The strain did not use ribose, sorbose, lactose, rhamnose, sucrose, trehalose, melezitose, raffinose, cellulose, xylan, salicin, dulcitol, inositol, mannitole, sorbitol, ethanol, glycerol, fumarate, lactate, malate, pyruvate, and succinate. Major products of strain WB4^{T} in PYG medium after 48 h incubation were propionate (14.0 mmol l^{-1}) and acetate (7.7 mmol l^{-1}) with a small amount of succinate (2.1 mmol l^{-1}). Gas was not produced. Vitamins including cobalamin were not required for growth or propionate production (Akasaka, 2004). Aesculin was hydrolyzed, but gelatin was not. Production of urease, hydrogen sulfide and indole were negative. The strain could not grow in the presence of 0.1% (w/v) bile salts, demonstrating that the strain was sensitive to bile.

Temperature range for growth was15-35ºC with an optimum at 30ºC. Even at 33ºC, the growth rate was significantly lower than that at 30ºC, and the strain could not grow at 37ºC. pH range for growth was 5.0-7.6 with an optimum at pH 6.6. NaCl concentration range for growth was 0-0.5% (w/v) in PYG medium, but the growth rate in the presence of more than 0.3% (w/v) NaCl was much lower than that in the absence of NaCl.

The predominant CFAs of strain WB4^T were anteiso-$C_{15:0}$ (30.8%), $C_{15:0}$, (19.0%), anteiso-3-OH $C_{17:0}$ (17.9%), and iso-3OH $C_{17:0}$ (6.2%). The major respiratory quinone of strain WB4^T was MK-8(H_4). The G + C content of genomic DNA of the strain was 39.3 ± 1.0 mol%.

An almost-complete 16S rDNA sequence of strain WB4^T assigned the strain to the phylum *Bacteroidetes* (Garrity & Holt, 2001). The closest relative of strain WB4^T found in GenBank was an uncultured bacterial clone (118ds10) from downstream of a collapsed equine manure pile with a 16S rRNA gene sequence similarity of 99.7% (Simpson *et al*., 2004). The second (similarity of 97.2%) to fifth closest (95.1%) relatives were all environmental clones (Moissl *et al*., 2002; Harris *et al*., 2004), with which strain WB4^T formed a distinct cluster (Fig. 2). The closest recognized species to strain WB4^T were *Dysgonomonas capnocytophagoides* CCUG 17996^T (formerly CDC group DF-3) (Hofstad *et al*., 2000) (90.9%), *Dysgonomonas mossii* CCUG 43457^T (Lawson *et al*., 2002) (89.8%) and *Bacteroides merdae* AY 169416 (Johnson *et al*., 1986) (89.2%). *Tannerella forsythensis* JCM 10827^T (formerly *Bacteroides forsythus*) (Sakamoto *et al*., 2002) and *Bacteroides fragilis* ATCC 25285^T (Holdeman *et al*., 1984) were the next most closely related species, with sequence similarities of 87.4% and 86.6%, respectively (Fig. 2).

Some characteristics of strain WB4^T and the three most closely related species are compared in Table 1. Cells of these species are commonly Gram-negative, non-motile and

non-spore-forming rods. *D. capnocytophagoides* and *D. mossii* are facultative anaerobes isolated from human clinical specimens and grow well aerobically and anaerobically at 35-37ºC (Hofstad *et al*., 2000; Lawson *et al*., 2002). *B. merdae* is a strict anaerobe isolated from human feces and has an optimum temperature at 37ºC (Johnson *et al*., 1986). The other two related species discussed above are derived from deep periodontal pockets of humans (*T. forsythensis*) (Sakamoto *et al*., 2002) or various types of human clinical specimens (*B. fragilis*) (Holdeman *et al*., 1984). Both are strict anaerobes and have optimum growth temperatures of about 37ºC. Thus, species related to strain WB4^T are mainly isolates from humans and other warm-blooded animals (Paster *et al*., 1994).

Strain WB4^T was a strict anaerobe and had an optimum growth temperature of 30ºC. Growth at 33ºC was much slower than that at 30ºC and the strain could not grow at 37ºC. The strain was isolated from irrigated rice-field soil, where daily mean soil temperature never exceeds 25ºC even in summer. Thus, strain WB4^T inhabits a distinctly different environment from those of related species, presumably reflected in its temperature dependence. Similarly, its closest relatives other than *T. forsythensis* are resistant to ox bile or bile salts (Holdeman *et al*., 1984; Johnson *et al*., 1986; Hofstad *et al*., 2000; Lawson *et al*., 2002; Sakamoto *et al*., 2002), whereas strain WB4^T was highly sensitive to bile salts.

Major end products from glucose of *D. capnocytophgoides* are propionate, lactate and

succinate (Hofstad *et al*., 2000) and those of *B. merdae* are succinate and acetate with a trace amount of propionate (Johnson *et al*., 1986) (Table 1). Furthermore, *T. forsythensis* produces various acids such as acetate, butyrate, isovalerate, propionate, phenylacetate and succinate (Sakamoto *et al*., 2002) and *B. fragilis* usually produces acetate and succinate as major products with propionate as a minor product (Holdeman *et al*., 1984; Moore *et al*., 1994). Strain WB4^T produced propionate and acetate at a ratio of 2:1 as the major products from glucose together with a smaller amount of succinate. Thus, although propionate and succinate seem to be common end-products for these related organisms, their physiological characteristics of their fermentative metabolism differ.

The G + C content of the genomic DNA of strain WB4^T (39.3 ± 1.0%) was similar to those of *D. capnocytophagoides* (38%) (Hofstad *et al*., 2000) and *D. mossii* (38.5%) (Lawson *et al*., 2002), but was somewhat lower than that of *B. merdae* (44%) (Johnson *et al*., 1986) (Table 1). It is also lower than those of *T. forsythensis* (44-48%) (Sakamoto *et al*., 2002) and *B. fragilis* (41-44%) (Holdeman *et al*., 1984). The respiratory quinones of *B. merdae* are MK-9 and MK-10 and those of *T. forsythensis* and *Bacteroides* species are commonly MK-10 and MK-11(Sakamoto *et al*., 2002). Thus, strain WB4^T has a distinctly different quinone composition from those of related species.

The CFA profiles of strain WB4^T and related species are presented in Table 2. Anteiso-$C_{15:0}$,

iso-$C_{15:0}$, iso-3-OH $C_{17:0}$ and $C_{16:0}$ are the major CFAs in *Bacteroides* species (Miyagawa *et al*., 1979; Moore *et al*., 1994). The overall CFA profiles of *B. merdae*, *T. forsythensis* and *B. fragilis* are generally consistent with this, although some of these signature fatty acids are only minor components in *B. merdae* and *T. forsythensis*. *D. capnocytophagoides* also has anteiso-$C_{15:0}$ as a dominant CFA (19.6%), but its profile is rather different from the other three species in having iso-$C_{14:0}$ (19.8%) and iso-3OH $C_{16:0}$ (12.3%) as predominant CFAs. Strain WB4^T has also anteiso-$C_{15:0}$ (30.8%) as the predominant fatty acid, but its overall profile is significantly different from those of any related species. Fatty acids such as iso-$C_{15:0}$, iso-3OH $C_{17:0}$ and $C_{16:0}$ are relatively minor components in strain WB4^T, whereas anteiso-3-OH $C_{17:0}$ (17.9%) and $C_{15:0}$ (19.0%) are major components. Anteiso-3-OH $C_{17:0}$ is also present in *B. merdae* and *T. forsythensis*, but it is only a minor component, and it is not found in *B. fragilis* or *D. capnocytophagoides*. The predominance of $C_{15:0}$ (19.0%) in strain WB4^T is also unique among the related species except for *D. capnocytophagoides*.

16S rRNA gene sequence similarities (about 90%) between strain WB4^T and the related recognized species suggested that strain WB4^T should be classified into a novel taxon. The data reported above demonstrate that the ecological, physiological and chemotaxonomic characteristics of strain WB4^T were clearly different from those of other related species. The closest relative to strain WB4^T on the database was an environmental clone isolated in the USA, with a sequence similarity of 99.7% (Simpson *et al*., 2004). The second most closely

related clone (similarity of 97.2%) was isolated from a cold sulfidic marsh in Germany (Moissl *et al*., 2002) and the third closest (96.3%) was isolated from a *Gallionella* community located on Boulder Creek, Co, USA (Harris *et al*., 2004). Other relatives with sequence similarities higher than about 95% were all environmental clones, with which strain WB4^T formed a distinct cluster (Fig. 2). These data suggest that an unknown bacterial linage closely related to strain WB4^T might be widespread and numerous in natural environments.

Based on the data presented, we propose here a novel genus, *Paludibacteroides* gen. nov., as *Paludibacter propionicigenes* sp. nov. as the type species.

Description of *Paludibacter* gen. nov.

Paludibacter (Pa.lu.di.bac'ter. L. n. *palus, -udis* a swamp, marsh; N.L. masc. n. *bacter* a rod; N.L. masc, n. *Paludibacter* rod living in swamps).

Cells are Gram-negative, non-spore-forming, non-motile, short rods. Strictly anaerobic. Chemo-organotroph. Optimum growth temperature is 30ºC. Oxidase, catalase and nitrate-reducing activities are negative. Species utilizes various sugars and produce acetate and propionate as major fermentation end-products with succinate as a minor product. Major cellular fatty acids are anteiso-$C_{15:0}$, $C_{15:0}$, and anteiso-3-OH $C_{17:0}$. The major respiratory quinone is MK-8(H_4). The genomic DNA G+C content of the type species is 39.3 mol%. The

type species is *Paludibacter propionicigenes*.

Description of *Paludibacter propionicigenes* sp. nov.

(pro.pi.on.i.ci'ge.nes. N.L. n. *acidum propionicum* propionic acid; Gr. v. *gennao* to produce; N.L., part. adj. *propionicigenes* propionic acid-producing)

Has the following properties in addition to those given for the genus. Cells are 0.5-0.6 µm in diameter and 1.3-1.7 µm in length. Ends of cells are usually round to slightly tapered and elongated cells are occasionally formed both singly and in chains of the short cells. Spherical cells sometimes occur after storage of slant cultures at 4ºC. Propionate and acetate are produced as major fermentation products from glucose at a ratio of 2:1. Succinate is also produced as a minor product. Grows in pH 5.0-7.6 (optimum at pH 6.6) and 15-35ºC (optimum at 30ºC). Even at 33ºC, growth is significantly slower than that at 30ºC. Does not grow at 37ºC. NaCl concentration range for growth is 0-0.5% (w/v) in PYG medium. Utilizes arabinose, xylose, cellobiose, fructose, galactose, glucose, mannose, maltose, melebiose, glycogen and soluble starch as growth substrates and produces acids. Does not use ribose, sorbose, lactose, rhamnose, sucrose, trehalose, melezitose, raffinose, cellulose, xylan, salicin, dulcitol, inositol, mannitole, sorbitol, ethanol, glycerol, fumarate, lactate, malate, pyruvate, and succinate. Aesculin is hydrolyzed, but gelatin is not. Urease is negative. Hydrogen sulfide and indole are not produced. Does not grow in the presence of bile salts.

The type strain, WB4^T (= JCM 13257^T = DSM 17365 ^{T}), was isolated from rice plant residue in anoxic rice-field soil in Japan.

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Figure legends

Fig. 1. A phase-contrast photomicrograph of strain WB4^T grown anaerobically on a agar slant of PY4S medium. Bar, 10 μm.

Fig. 2. Neighbor-joining tree showing the phylogenetic relationship of strain WB4^T and related species in the *Bacteroidetes* 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 2% estimated difference in nucleotide sequence position. The sequence of *Escherichia coli*, which belongs to the *Gammaproteobacteria* (Garrity & Holt, 2001), was used as the outgroup.

Fig. 1

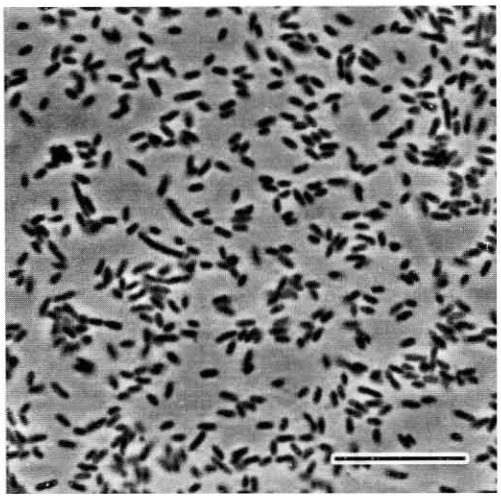

Fig. 2

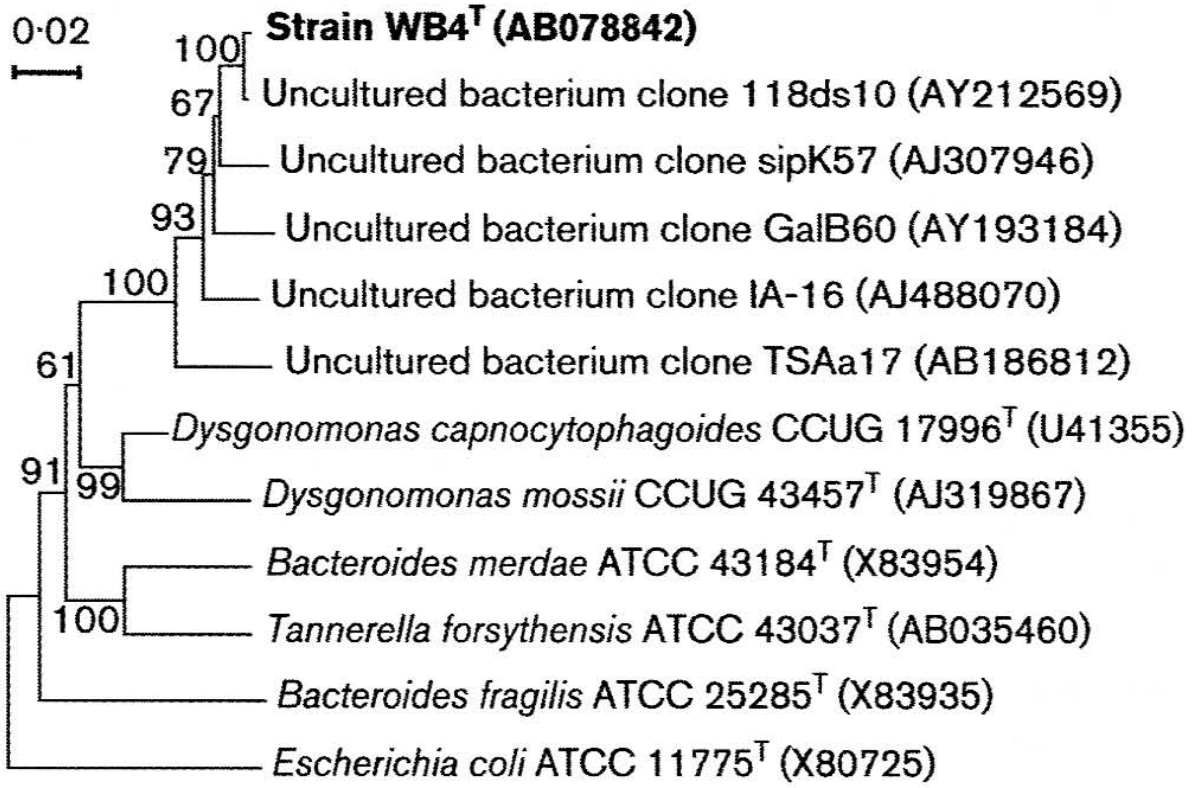
0·02
100
Strain WB4T (AB078842)
67
Uncultured bacterium clone 118ds10 (AY212569)
79
Uncultured bacterium clone sipK57 (AJ307946)
93
Uncultured bacterium clone GalB60 (AY193184)
100
Uncultured bacterium clone IA-16 (AJ488070)
61
Uncultured bacterium clone TSAa17 (AB186812)
Dysgonomonas capnocytophagoides CCUG 17996T (U41355)
91
99
Dysgonomonas mossii CCUG 43457T (AJ319867)
Bacteroides merdae ATCC 43184T (X83954)
100
Tannerella forsythensis ATCC 43037T (AB035460)
Bacteroides fragilis ATCC 25285T (X83935)
Escherichia coli ATCC 11775T (X80725)

Table 1. Some characteristics of strain WB4^T and related species. Strain: 1. WB4^T; 2, *Dysgonomonas capnocytophagoides* CCUG 17996^T (data from Hofstad *et al*., 2000); 3, *Dysgonomonas mossi* CCUG 43457^T (Lawson *et al*., 2002); 4, *Bacteroides merdae* JCM 9497^T (Johnson *et al*., 1986). +, positive reaction, -, negative reaction; n.d.. no data available, A, acetate; P or p, propionate; L, lactate; S or s, succinate (lowercase letters, minor products). All strains were positive for aesculin hydrolysis. All strains were negative for gelatin hydrolysis, uerase and oxidase.

Characteristics	1	2	3	4
Source	irrigated rice field soil	human clinical specimen	human clinical specimen	human feces
Cell shape	short rods	cocobacilli to short rods	cocobacilli to short rods	short to long rods
Aerobic growth	-	+	+	-
Growth in bile	-	+	+	+
Catalase	-	+	-	-
Oxidase	-	-	-	-
Aesculin hydrolysis	+	+	+	+
Gelatin hydrolysis	-	-	-	-
Indole production	-	+	+	-
Urease	-	-	-	-
H_2S production	-	-	n.d.	+
Nitrate reduction	-	-	-	n.d.
Products from glucose	A, P, s	P, L, S	n.d.	A, S, p
Optimum temp (ºC)	30	35-37	35-37	37
Growth at 37ºC	-	+	+	+
DNA G+C content (mol%)	39.3	38	38.5	43-46

Table 2. Cellular fatty acids composition (%) of strain WB4^T and related species.
Strain: 1, WB4^T; 2, *Dysgonomonas capnocytophagoides* CCUG 17996^T (data from Hofstad *et al*., 2000); 3, *Bacteroides merdae* JCM 9497^T (Sakamoto *et al*., 2002); 4. *Tannerella forsythensis* JCM 10827^T (Sakamoto *et al*., 2002); 5. *Bacteroides fragilis* NCTC 9343^T (Miyagawa *et al*., 1979).
Data for *Dysgonomonas mossi* were not available. -, not detected.

Fatty acid	1	2	3	4	5
Saturated straight-chain:					
$C_{14:0}$	2.8	2.4	0.8	1.5	-
$C_{15:0}$	19.0	11.3	0.6	-	2.0
$C_{16:0}$	4.9	3.4	3.9	9.1	14.6
$C_{17:0}$	0.4	-	-	-	-
$C_{18:0}$	1.4	-	1.1	3.3	-
Unsaturated straight-chain:					
$C_{15:1}$	-	3.4	-	-	-
$C_{16:1}$	-	4.7	-	-	-
$C_{18:1}$	-	-	6.5	2.7	-
Hydroxy acids:					
iso-3-OH $C_{15:0}$	1.1	-	-	1.1	-
3-OH $C_{15:0}$	2.6	-	-	-	-
iso-3-OH $C_{16:0}$	1.1	12.3	-	-	-
3-OH $C_{16:0}$	2.4	4.6	5.6	15.8	8.0
iso-3-OH $C_{17:0}$	6.2	3.3	23.5	7.7	18.1
anteiso-3-OH $C_{17:0}$	17.9	-	5.6	4.2	-
Satureted branched-chain:					
iso-$C_{13:0}$	0.3	9.5	-	-	-
iso-$C_{14:0}$	0.3	19.8	-	-	-
iso-$C_{15:0}$	1.1	2.7	6.4	1.7	10.5
anteiso-$C_{15:0}$	30.8	19.6	40.1	53.0	41.5
iso-$C_{17:0}$	0.3	-	-	-	-
anteiso-$C_{17:0}$	0.8	-	0.7	-	3.5