Paludibacter propionigenes 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, Hiroshi Akasaka,† Daisuke Suzuki and Katsuji Ueki

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

Correspondence

Atsuko Ueki

uatsuko@tds1.tr.yamagata-u.ac.jp

A strictly anaerobic, propionate-producing bacterial 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 30 °C. Oxidase, catalase and nitrate-reducing activities were negative. The major cellular fatty acids were anteiso-C_{15:0}, C_{15:0} and anteiso-C_{17:0} 3-OH. Menaquinone MK-8(H₄) 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 phylum '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 propionigenes* gen. nov., sp. nov., is proposed to accommodate it. The type strain is WB4^T (=JCM 13257^T=DSM 17365^T).

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 the 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 hydrogen for methanogenesis (Cicerone & Oremland, 1988; Boone, 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, 2004). In this study, we have characterized one of the isolates (strain WB4^T) from plant residue samples (Akasaka *et al.*, 2003a, 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 were 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 irrigated rice-field soil in the Shonai Branch of the Yamagata Agricultural Experimental Station (Fujishima-machi, Yamagata, Japan) during the

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

Abbreviation: CFA, whole-cell fatty acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WB4^T is AB078842.

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, 2004).

The strain was cultivated anaerobically at 30 °C unless otherwise stated by using peptone/yeast extract (PY) medium as basal medium with oxygen-free, 95 % N₂/5 % CO₂ 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 OD₆₆₀.

Growth of the strain under aerobic conditions was examined by plate culture on nutrient agar (Nissui Pharmacy) and PY4S agar modified to exclude Na₂CO₃, cysteine hydrochloride and sodium resazurin. Spore formation was assessed by observation of cells after Gram-staining and by growth in PYG medium of cells exposed to 80 °C 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 (Oxoid) (0.1–0.5 %, w/v) to PYG medium (Lawson *et al.*, 2002). Fermentation products were analysed by GC or HPLC as described previously (Ueki *et al.*, 1986; Akasaka *et al.*, 2003a). Other characterization was performed according to the methods 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 analysed by GC [HP6890 (Hewlett Packard) or G-3000 (Hitachi)] equipped with an HP Ultra2 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). The TSBA40 microbial identification library (MIDI Inc.) was used to confirm identification. Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analysed by using a mass spectrometer (JMS-SX102A; JEOL). Genomic DNA was extracted according to the method of Kamagata & Mikami (1991). Extracted DNA was digested with P1 nuclease by using a Yamasa GC kit (Yamasa shoyu) and its G+C content was measured by HPLC (Hitachi L-7400) equipped with a μ Bondpack C18 column (3.9 × 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 neighbour-joining method (Saitou & Nei, 1987) by using the CLUSTAL W program (Thompson *et al.*, 1994). All gaps and unidentified base positions in the alignments were excluded before sequence assembly.

Cells of strain WB4^T were Gram-negative, short rods, 0.5–0.6 μ m in diameter and 1.3–1.7 μ m 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 developed after storage of slant cultures for 1–2 months at 4 °C. Cells were non-motile as observed under phase-contrast microscopy. The strain could not grow in air on either PY4S or 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 30 °C; however, inoculation from older cultures sometimes significantly delayed growth. Spore formation was not observed and cells treated at 80 °C 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

Fig. 1. Phase-contrast photomicrograph of cells of strain WB4^T grown anaerobically on an agar slant of PY4S medium. Bar, 10 μ m.

Table 1. Some characteristics of strain WB4^T and related species

Strains: 1, WB4^T; 2, *Dysgonomonas capnocytophagoides* CCUG 17996^T (data from Hofstad *et al.*, 2000); 3, *Dysgonomonas mossii* CCUG 43457^T (Lawson *et al.*, 2002); 4, *Bacteroides merdae* JCM 9497^T (Johnson *et al.*, 1986). +, Positive, –, negative; ND, no data available. All strains were positive for aesculin hydrolysis. All strains were negative for gelatin hydrolysis, urease and oxidase.

Characteristic	1	2	3	4
Source	Irrigated rice-field soil	Human clinical specimen	Human clinical specimen	Human faeces
Cell shape	Short rods	Coccobacilli to short rods	Coccobacilli to short rods	Short to long rods
Aerobic growth	–	+	+	–
Growth in bile	–	+	+	+
Catalase	–	+	–	–
Indole production	–	+	+	–
H ₂ S production	–	–	ND	+
Nitrate reduction	–	–	–	ND
Products from glucose*	A, P, s	P, L, S	ND	A, S, p
Optimum growth temperature (°C)	30	35–37	35–37	37
Growth at 37 °C	–	+	+	+
DNA G+C content (mol%)	39.3	38	38.5	43–46

*A, Acetate; L, lactate; P/p, propionate; S/s, succinate. Lower-case letters indicate minor products.

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, the physiological characteristics of their fermentative metabolism differ.

The G+C content of the genomic DNA of strain WB4^T (39.3 ± 1.0 mol%) was similar to those of *D. capnocytophagoides* (38 mol%) (Hofstad *et al.*, 2000) and *D. mossii* (38.5 mol%) (Lawson *et al.*, 2002), but was somewhat lower than that of *B. merdae* (44 mol%) (Johnson *et al.*, 1986) (Table 1). It is also lower than those of *T. forsythensis* (44–48 mol%) (Sakamoto *et al.*, 2002) and *B. fragilis* (41–44 mol%) (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-C_{17:0} 3-OH and C_{16:0} are the major CFAs found 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-C_{16:0} 3-OH (12.3%) as predominant CFAs. Strain WB4^T also has 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-C_{17:0} 3-OH and C_{16:0} are relatively minor components in strain WB4^T, whereas anteiso-C_{17:0} 3-OH (17.9%) and C_{15:0} (19.0%) are major components. Anteiso-C_{17:0} 3-OH 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 related recognized species suggested that strain WB4^T should be classified within a novel taxon. The data reported above demonstrate that the ecological, physiological and chemotaxonomic characteristics of strain WB4^T are clearly different from those of other related species. The closest relative of 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 lineage 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, *Paludibacter* gen. nov., with *Paludibacter propionigenes* sp. nov. as the type species.

Table 2. Cellular fatty acid composition (%) of strain WB4^T and related species

Strains: 1, WB4^T; 2, *D. 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 mossii* 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-C _{15:0} 3-OH	1.1	—	—	1.1	—
C _{15:0} 3-OH	2.6	—	—	—	—
iso-C _{16:0} 3-OH	1.1	12.3	—	—	—
C _{16:0} 3-OH	2.4	4.6	5.6	15.8	8.0
iso-C _{17:0} 3-OH	6.2	3.3	23.5	7.7	18.1
anteiso-C _{17:0} 3-OH	17.9	—	5.6	4.2	—
Saturated 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

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 30 °C. Oxidase, catalase and nitrate-reducing activities are negative. Species utilize 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-C_{17:0} 3-OH. The major respiratory quinone is MK-8(H₄). The genomic DNA G+C content of the type species is 39.3 mol%. The type species is *Paludibacter propionigenes*.

Description of *Paludibacter propionigenes* sp. nov.

Paludibacter propionigenes (pro.pi.on.i.ci'ge.nes. N.L. n. *acidum propionicum* propionic acid; Gr. v. *gennai* to

produce; N.L. part. adj. *propionigenes* 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 develop after storage of slant cultures at 4 °C. Propionate and acetate are produced as major fermentation products from glucose at a ratio of 2:1. Grows at pH 5.0–7.6 (optimum pH 6.6) and at 15–35 °C. Even at 33 °C, growth is significantly slower than that at 30 °C. Does not grow at 37 °C. NaCl concentration range for growth is 0–0.5 % (w/v) in PYG medium. Utilizes arabinose, xylose, cellobiose, fructose, galactose, glucose, mannose, maltose, melibiose, glycogen and soluble starch as growth substrates and produces acids. Does not use ribose, sorbose, lactose, rhamnose, sucrose, trehalose, melizitol, raffinose, cellulose, xylan, salicin, dulcitol, inositol, mannitol, sorbitol, ethanol, glycerol, fumarate, lactate, malate, pyruvate or succinate. Aesculin is hydrolysed, 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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