Propioniciclava tarda gen. nov., sp. nov., isolated from a methanogenic reactor treating waste from cattle farms

Yoshimichi Sugawara,¹ Atsuko Ueki,¹ Kunihiro Abe,¹ Nobuo Kaku,¹ Kazuya Watanabe² and Katsuji Ueki¹

Correspondence

Atsuko Ueki

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

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

²Exploratory Research for Advanced Technology (ERATO), JST, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

Two facultatively anaerobic bacterial strains, designated WR061^T and WR054, were isolated from rice-straw residue in a methanogenic reactor treating waste from cattle farms in Japan. The two strains were phylogenetically positioned close to one another and had almost the same phenotypic properties. Cells were Gram-reaction-positive, non-motile, non-spore-forming, irregular rods. Cobalamin (vitamin B₁₂) was required for growth. The strains utilized various carbohydrates, including hexoses and disaccharides, and produced acetate and propionate from these carbohydrates. Pentoses and polysaccharides were not utilized. They grew at 20–37 °C (optimum 35 °C) and pH 5.3–8.0 (optimum pH 6.8–7.5). Catalase and nitrate-reducing activities were detected. Aesculin was hydrolysed. The major cellular fatty acids were anteiso-C_{15:0} and C_{15:0} DMA, the major respiratory quinone was menaquinone MK-9(H₄) and the genomic DNA G + C content was 69.3–69.5 mol%. The diagnostic diamino acid in the peptidoglycan was *meso*-diaminopimelic acid. Phylogenetic analysis based on 16S rRNA gene sequences placed the strains in the phylum *Actinobacteria*. Both strains were remotely related to the species in the family *Propionibacteriaceae* and *Propionibacterium propionicum* JCM 5830^T was the most closely related type strain with a sequence similarity of 91.6 %. Based on phylogenetic, physiological and chemotaxonomic analyses, the two novel strains together represent a novel species of a new genus, for which the name *Propioniciclava tarda* gen. nov., sp. nov. is proposed. The type strain is WR061^T (=JCM 15804^T =DSM 22130^T).

Propionate is one of the major volatile fatty acids produced by fermentative bacteria during anaerobic decomposition of organic matter in methanogenic ecosystems. It often accumulates in methanogenic reactors, causing a pH decrease and an instability of the system (Dolfing, 1988; Ueki *et al.*, 1986). In this study, we describe a novel, facultatively anaerobic, propionate-producing bacterium that was isolated from a methanogenic reactor treating cattle waste.

Strain WR061^T (=JCM 15804^T =DSM 22130^T) and WR054 (=JCM 15805) were isolated by using the anaerobic roll-tube method (Holdeman *et al.*, 1977) from a sample of rice-straw residue obtained from a methanogenic reactor treating waste collected from cattle farms (housing up to 1000 cattle in total) in Betsukai-machi, Hokkaido,

Japan (Nishiyama *et al.*, 2009a, b; Ueki *et al.*, 2008, 2011). The reactor was of the vertical cylindrical type (1500 m³) and was operated at 35 °C. Rice straw is used as matting at the cattle farms and the spent straw, containing cattle faeces and urine, is thrown into the reactor and treated as waste. The rice-straw residue samples obtained from the reactor were washed several times with sterile anoxic diluent and homogenized in a Waring blender (10 000 r.p.m. for 10 min) under N₂ atmosphere. The homogenized samples were successively diluted (10-fold) under anaerobic conditions and used as inocula for anaerobic roll-tubes containing PY4S medium supplemented with a vitamin B mixture (PYV4S) (Akasaka *et al.*, 2004). Colonies that formed on the agar were picked at random after incubation for 2 weeks at 30 °C and about 50 isolates were obtained from a sample. Strains WR061^T and WR054 were picked from a roll-tube inoculated with a 10⁻⁴ diluted sample.

Both strains were cultivated anaerobically at 30 °C unless otherwise stated using peptone–yeast extract (PY) medium

Abbreviations: DMA, dimethylacetate; DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains WR061^T and WR054 are AB298731 and AB298730, respectively.

(Holdeman *et al.*, 1977) as the basal medium with oxygen-free mixed gas (N_2/CO_2 , 95:5) as the headspace, as described by Ueki *et al.* (2008). PY medium supplemented with (1^{-1}) 0.25 g each of glucose, cellobiose, maltose and soluble starch as well as 15 g agar (Difco) was designated PY4S agar and was used for maintenance of the strains in agar slants. PY liquid medium supplemented with the vitamin B mixture (PYV medium) or cobalamin (PYB_{12} medium) was used as a basal medium for cultivation under various conditions. PYB_{12} medium contained cyanocobalamin (cobalamin or vitamin B_{12}) as a sole vitamin, which was added at the same concentration ($10 \mu\text{g l}^{-1}$) as that in the vitamin B mixture (Akasaka *et al.*, 2004), and PYB_{12} medium containing 10 g glucose 1^{-1} (PYB_{12}G medium) was used for cultivation of the strains for various physiological and chemotaxonomic tests. Cells previously transferred at least twice in the medium containing the vitamin mixture or cobalamin were used as inocula for various cultivation experiments. Except for when pH range for growth was being determined, the pH of the media was adjusted to pH 7.4–7.5, since the strains preferred slightly alkaline conditions. Growth in liquid media was monitored by changes in OD_{660} . The reference type strain of the closest related species, *Propionibacterium propionicum* JCM 5830^T, was cultivated under the same conditions using the same media adjusted to pH 7.0.

Growth of the strains under aerobic conditions was examined as described previously (Ueki *et al.*, 2008). Spore formation was assessed by observation of cells after Gram staining and the production of thermotolerant cells was determined by cultivating heat-treated (80°C for 10 min) cells in PYB_{12}G liquid medium. The motility of cells was examined from both slant and liquid cultures using phase-contrast microscopy. Oxidase and nitrate-reducing activities were determined according to the methods described by Akasaka *et al.* (2003). Catalase activity was determined by bubble production from cells cultivated in liquid media, collected by centrifugation and mixed with a small amount of H_2O_2 solution (3 %, v/v) (Wilkins *et al.*, 1978). Growth was determined at $10\text{--}45^\circ\text{C}$ (at 5°C intervals and at 37°C), at pH 4.0, 5.3, 5.8, 6.8, 7.5, 8.0, 9.2 and 10.2 (values verified after autoclaving), and in the presence of 0–5 % (w/v) NaCl (at 1 % intervals and at 0.5 %) in PYB_{12}G medium. Bicine [*N,N*-bis(2-hydroxyethyl)glycine] (Good's buffer; Dotite) (20 mM) was used to adjust the media to pH 8.0 and above using N_2 gas as the headspace (Ueki *et al.*, 2008). Growth in liquid medium was monitored by changes in OD_{660} . Utilization of carbon sources was tested in PYB_{12} medium, each substrate being added at 10 g l^{-1} (for sugars and sugar alcohols) or 5 g l^{-1} for cellulose (filter paper) and xylan. Organic acids were added at final concentrations of 30 mM. Utilization of each substrate was determined from measuring growth (OD_{660}) (except xylan and filter paper) as well as by detecting fermentation products in the medium after cultivation. Fermentation products were analysed by GC as described previously (Ueki *et al.*, 1986). Production of urease,

hydrogen sulfide and indole as well as hydrolysis of aesculin and gelatin was tested according to the methods described by Holdeman *et al.* (1977). Bile sensitivity was determined in PYB_{12}G medium supplemented with 0.1–2.0 % (w/v) Oxgall (Difco). All experiments for biochemical characterization were performed in duplicate.

Strains WR061^T and WR054 grew poorly as colonies with slightly dry surfaces on PY4S agar slants. Cells were Gram-reaction-positive and non-motile and, when grown on PY4S agar, were irregular and wedge-shaped rods usually occurring in pairs or chains with irregular V-shaped cell arrangements (Fig. 1a). Cells were $0.5\text{--}0.8 \times 0.6\text{--}1.8 \mu\text{m}$ in size. Spore formation was not observed and cells did not grow after heat treatment. The strains grew aerobically on either PY4S or nutrient agar. Cell morphology when grown under aerobic conditions was the same as when grown under anaerobic conditions.

Growth of both strains in PY or PYG liquid medium was poor but the addition of B-vitamin mixture (PYVG) stimulated the growth. Cobalamin showed almost the same effect on growth, indicating that both strains required cobalamin for growth. Thus, physiological characteristics of the strains were determined in the presence of cobalamin (PYB_{12} medium). Growth rates of both strains, however, were rather low, even in the presence of the vitamin mixture or cobalamin. Increase in the concentration of cobalamin (up to $100 \mu\text{g l}^{-1}$) did not improve growth. When cells grown on PY4S agar slants were directly inoculated to PYB_{12}G liquid medium, the cultures took 12 days to reach early stationary phase, whereas this time was shortened to 3–4 days when cells cultivated on PYV4S slants were used as an inoculum. Cells grown in PYV4S agar were slender and longer ($3\text{--}6 \mu\text{m}$) than those grown in PY4S agar and often formed irregular and

Fig. 1. Phase-contrast photomicrographs of cells of strain WR061^T grown on PY4S agar (a) and PYV4S agar (b). Bar, $5 \mu\text{m}$.

crescent-shaped arrangements. Mycelial forms were not observed (Fig. 1b).

The temperature range for growth was 20–37 °C (optimum 35 °C) with very weak growth occurring at 37 °C. The strains grew at pH 5.3–8.0 and, although the growth rates were almost the same at the pH range of 6.8–7.5, the highest growth yield was obtained at pH 7.5 (based on OD₆₆₀). The final pH of the media after growth with glucose was pH 4.8–4.9. The highest growth rate (μ) obtained under the optimum pH and temperature was 0.05–0.07 h⁻¹. The NaCl concentration range for growth was 0–0.5 % (w/v) NaCl; concentrations of 0.5 % (w/v) NaCl and above strongly suppressed growth. Aesculin was hydrolysed. Catalase activity was detected in cells grown in PY4S agar slants or in PYB₁₂G medium. Nitrate-reducing activity was detected. The strains did not tolerate bile, even 0.1 % (w/v) Oxgall. Other physiological characteristics are given in the species description.

The strains utilized fructose, galactose, glucose, cellobiose, lactose and salicin. The strains also utilized mannose, maltose, melibiose, sucrose, trehalose, melezitose, raffinose, mannitol, amygdalin, aesculin, pyruvate and lactate; however, growth on these substrates was delayed compared with the substrates listed previously. The strains did not utilize pentoses, polysaccharides or sugar alcohols. Other substrates tested but not used by the strains are shown in the species description.

Strain WR061^T produced acetate (1.1 mmol l⁻¹) and propionate (2.4 mmol l⁻¹) from PYB₁₂ medium and produced acetate (16.0 mmol l⁻¹) and propionate (25.5 mmol l⁻¹) with a trace amount of succinate (1.5 mmol l⁻¹) from PYB₁₂G medium. Almost the same amounts of products were formed from other substrates utilized (the molar ratio of acetate and propionate being ~1:2). When grown on lactate (30 mM), acetate (14.3 mmol l⁻¹) and propionate (34.4 mmol l⁻¹) were detected in the medium. Strain WR054 produced almost the same amounts of products from various substrates. The requirement for exogenous cobalamin to grow and produce propionate has been reported for fermentative bacterial species isolated from irrigated rice-field soil (Akasaka *et al.*, 2003) and a methanogenic reactor (Ueki *et al.*, 2008, 2011). Cobalamin is essential for methylmalonyl-CoA isomerase to catalyse propionate production in the methylmalonyl-CoA pathway (Roth *et al.*, 1996).

DNA samples extracted from cells of the novel strains and the almost-complete 16S rRNA gene sequences were PCR amplified using the primer pair 8f and 1546r as described previously (Akasaka *et al.*, 2003). The PCR-amplified 16S rRNA genes were sequenced by using an ABI Prism BigDye Terminator Cycle Sequencing ready reaction kit and an ABI Prism 3730 automatic DNA sequencer (Applied Biosystems). Multiple alignments of the amplified sequences with reference sequences in GenBank were performed with the BLAST program (Altschul *et al.*, 1997). A phylogenetic tree was reconstructed by using the

neighbour-joining method (Saitou & Nei, 1987) in the CLUSTAL W program (Thompson *et al.*, 1994) and the maximum-likelihood method (DNAML) in the PHYLIP 3.66 package (Felsenstein, 2006). All gaps and unidentified base positions in the alignments were excluded before sequence assembly. The sequence length produced was 1453 bp for strain WR061^T and 1448 bp for strain WR054. There were two mismatches between the two sequences and the sequence of WR061^T had one gap. 16S rRNA gene sequence similarity between the two strains was 99.86 %. Phylogenetic analysis based on 16S rRNA gene sequences placed both strains in the phylum *Actinobacteria*. They were affiliated with the family *Propionibacteriaceae* in the order *Actinomycetales* (Garrity & Holt, 2001). The species most closely related to both strains was *P. propionicum* (Charfreitag *et al.*, 1988; Cummins & Johnson, 1986; Gerencser & Slack, 1967; Schaal, 1986), originally isolated from a case of human lacrimal canalculitis, with a 16S rRNA gene sequence similarity of 91.6 % between *P. propionicum* JCM 5830^T and the two novel strains. *Propionibacterium avidum* ATCC 25577^T (Cummins & Johnson, 1986) was the next closely related type strain (sequence similarity 91.2 %). Both novel strains formed a distinct branch in the phylogenetic tree reconstructed using the neighbour-joining method (Fig. 2). When evaluated by using the maximum-likelihood method (data not shown), the tree topology was essentially the same as that obtained with the neighbour-joining method.

The two novel strains and the type strain of their closest relative (*P. propionicum* JCM 5830^T) were cultivated under the same conditions. Their cellular fatty acids were converted to methyl esters according to the method of Miller (1982) and analysed by GC (HP6890; Hewlett Packard or G-3000; Hitachi) equipped with a HP Ultra 2 column. Fatty acid methyl esters were identified from equivalent chain-lengths (ECL) (Miyagawa *et al.*, 1979) according to the protocol of TechnoSuruga (Shimidu, Japan) (Moore *et al.*, 1994). Both novel strains had almost the same fatty acid composition (Table 1). The major fatty acids of strains WR061^T and WR054 were anteiso-C_{15:0} (~34.0 % for both strains), C_{15:0} dimethylacetal (DMA) (10 and 14.0 %, respectively) and anteiso-C_{15:0} DMA (6.7 and 9.2 %). The total percentages of C_{15:0} compounds in strains WR061^T and WR054 were 72.6 and 70.0 %, respectively. Various DMA-type components were detected; the total amounts of DMA type components in strains WR061^T and WR054 were 38.3 and 31.8 %, respectively. Unsaturated fatty acids were not detected. The major fatty acids of the closest relative, *P. propionicum* JCM 5830^T, were anteiso-C_{15:0} (32.5 %), iso-C_{15:0} (31.0 %) and C_{19:0} cyclo DMA (ECL 19.322) (20.3 %). The presence of branched-chain C_{15:0} compounds as major components was common to the three strains; however, the overall profile of *P. propionicum* JCM 5830^T was significantly different from those of the novel strains; the presence of cyclo-type fatty acids has not been reported in *P. propionicum* (Charfreitag *et al.*, 1988; Cummins & Moss, 1990; Kusano *et al.*, 1997; Schaal, 1986).

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of novel strains WR061^T and WR054, other species in the family *Propionibacteriaceae* and some representative type species of other families in the order *Actinomycetales*. Bootstrap values >70% (based on 1000 replications) are shown at branch nodes. *Acidimicrobium ferrooxidans* JCM 1546^T was used as the outgroup. The tree topology evaluated by using the maximum-likelihood method (data not shown) was almost the same as that obtained with the neighbour-joining method. Bar, 0.01 substitutions per nucleotide position.

Genomic DNA of strains WR061^T, WR054 and *P. propionicum* JCM 5830^T was extracted according to the method described by Akasaka *et al.* (2003) and digested with P1 nuclease by using a YAMASA GC kit (Yamasa Shoyu). The G+C contents of their genomic DNA were measured by HPLC (Hitachi L-7400) equipped with a μ Bondapak C18 column (3.9 $\times$ 300 mm; Waters). The genomic DNA G+C content of strains WR061^T and WR054 were 69.5 and 69.3 mol%, respectively, and that of *P. propionicum* JCM 5830^T was 65.3 mol% (consistent with the values of 63–65% reported for this species; Schaal,

1986). Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analysed by using a mass spectrometer (JMS-SX102A; JEOL). Menaquinone MK-9(H₄) was the major respiratory quinone of the novel strains, which was the same as that of *P. propionicum* (Charfreitag *et al.*, 1988). The diagnostic cell-wall diamino acids of strains WR061^T, WR054 and *P. propionicum* JCM 5830^T were analysed according to the method described by Akasaka *et al.* (2003). The amino acid of the two novel strains was *meso*-diaminopimelic acid (DAP), while that of *P. propionicum* JCM 5830^T was LL-DAP (consistent with that reported for this species; Schaal, 1986).

Some differential cellular and physiological characteristics of *P. propionicum* JCM 5830^T, including cell morphology, cobalamin requirement and substrate utilization, were determined under the same conditions used for the characterization of strains WR061^T and WR054. *P. propionicum* JCM 5830^T produced dense and rough colonies with greyish surfaces on PY4S agar slants. The colonies were composed of aggregates of distinctly long and branched, filamentous cells as reported in the description of this species (Schaal, 1986). Filamentous assemblages of cells did not occur in cultures of strains WR061^T and WR054. *P. propionicum* JCM 5830^T did not require cobalamin for growth and produced acetate and propionate (~1:2) from glucose irrespective of the presence or absence of cobalamin. *P. propionicum* JCM 5830^T was also negative for catalase activity, whereas strains WR061^T and WR054 were catalase-positive. Differences in other characteristics including substrate utilization are shown in Table 2.

The significantly low 16S rRNA gene sequence similarities between the two novel strains and their closest relatives, as well as the distinct differences in their cellular, physiological and chemotaxonomic characteristics, clearly indicate that strains WR061^T and WR054 represent a novel species of a new genus, for which the name *Propioniclelava tarda* gen. nov., sp. nov. is proposed.

Table 1. Cellular fatty acid composition (%) of strains WR061^T and WR054 and their closest relative, *Propionibacterium propionicum* JCM 5830^T

Strains: 1, WR061^T; 2, WR054; 3, *Propionibacterium propionicum* JCM 5830^T. —, Not detected; DMA, dimethylacetal. All data were obtained in this study.

Fatty acid	1	2	3
iso-C _{14:0}	2.3	1.6	—
C _{14:0}	0.4	0.6	2.3
iso-C _{15:0}	7.5	8.6	31.0
anteiso-C _{15:0}	33.7	34.6	32.5
C _{15:0}	3.5	4.2	9.2
iso-C _{15:0} DMA	4.7	5.6	—
anteiso-C _{15:0} DMA	9.2	6.7	—
C _{15:0} DMA	14.0	10.3	—
C _{16:0}	0.4	1.6	2.3
C _{16:0} DMA	3.2	5.0	—
anteiso-C _{17:0}	—	—	1.1
C _{17:0}	0.5	1.0	1.4
C _{17:0} DMA	7.2	4.2	—
C _{19:0} cyclo 9,10 DMA*	—	—	20.3

*Equivalent chain-length=19.322.

Table 2. Characteristics of strains WR061^T and WR054 and their closest relative, *Propionibacterium propionicum* JCM 5830^T

Strains: 1, WR061^T; 2, WR054; 3, *Propionibacterium propionicum* JCM 5830^T. All data were obtained in this study using the same methods for cultivation and analyses. I, irregular rods; S, slender rods; P, pleomorphic, filamentous rods; +, positive; w, weakly positive; –, negative.

Characteristic	1	2	3
Isolation source	Methanogenic reactor	Methanogenic reactor	Human
Cell morphology	I, S	I, S	P
Cobalamin requirement	+	+	–
Growth in PY(B ₁₂) medium without substrates	w	w	+
Catalase	+	+	–
Aesculin hydrolysis	+	+	–
Acid production from			
Cellobiose	+	+	–
Melibiose	+	+	w
Amygdalin	+	+	–
Aesculin	+	+	–
Salicin	+	+	–
Pyruvate	w	w	+
Lactate	w	w	+
DNA G + C content (mol%)	69.5	69.3	65.3
Diamino acid	meso-DAP	meso-DAP	LL-DAP

Description of *Propioniciclava* gen. nov.

Propioniciclava (Pro.pi.on.i.ci.clav'a. N.L. n. *acidum* *propionicum* propionic acid; N.L. fem. n. *clava* club; N.L. fem. *Propioniciclava* propionic acid-producing club).

Cells are facultatively anaerobic, Gram-reaction-positive, non-motile, non-spore-forming, irregular rods. Chemo-organotrophic. Ferments various carbohydrates and produces acetate and propionate from glucose. The diagnostic diamino acid in the peptidoglycan is *meso*-DAP. The major respiratory quinone is menaquinone MK-9(H₄). The genomic DNA has a high G + C content. The type species of the genus is *Propioniciclava tarda*.

Description of *Propioniciclava tarda* sp. nov.

Propioniciclava tarda (tar'da L. fem. adj. *tarda* slow or inactive, referring to the slow growth of the type strain).

Possesses the following characteristics in addition to those described for the genus. Cobalamin (vitamin B₁₂) is an essential requirement for growth. In the absence of cobalamin, cells are irregular rods with tapered ends (0.5–0.8 × 0.6–1.8 µm) and occur singly or in short chains. In the presence of cobalamin, cells are slender irregular rods (3–6 µm long) with crescent-shaped arrangements. Growth is very slow, even in the presence of cobalamin. Grows under aerobic conditions. Grows at 20–37 °C (optimum 35 °C) and pH 5.3–8.0, preferring slightly alkaline conditions (optimum pH 7.5). Addition of NaCl to the medium inhibits growth. Positive for catalase and nitrate-reducing activities. Aesculin is hydrolysed. Oxidase, indole and hydrogen sulfide are not produced. Starch is not hydrolysed. Sensitive to bile. Utilizes fructose, galactose, glucose, cellobiose, lactose and salicin as preferable

substrates. The final pH of media after growth with glucose is 4.8–4.9. Produces acetate and propionate with a small amount of succinate. Mannose, maltose, melibiose, sucrose, trehalose, melezitose, raffinose, mannitol, amygdalin, aesculin, pyruvate and lactate are also utilized with slow growth. Arabinose, ribose, xylose, rhamnose, sorbose, carboxymethylcellulose (CMC), cellulose (cellulose powder and filter paper), glycogen, inulin, starch, xylan, pectin, dulcitol, inositol, sorbitol, glycerol, ethanol, fumarate, malate and succinate are not utilized. Anteiso-C_{15:0} and C_{15:0} DMA are the major cellular fatty acids; C_{15:0} and DMA type compounds are the major fatty acid constituents.

The type strain, WR061^T (=JCM 15804^T =DSM 22130^T), was isolated from a sample of rice-straw residue in a methanogenic reactor treating waste from cattle farms. The DNA G + C content of the type strain is 69.3–69.5 %.

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