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

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Two facultatively anaerobic bacterial strains, designated WR061T 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-C15:0 and C15:0 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 5830T 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 WR061T (＝JCM 15804T＝DSM 22130T).

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 WR061T (=JCM 15804T＝DSM 22130T) 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 was 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 WR061T 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.
Propioniclava tarda gen. nov., sp. nov.

(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 (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 was used for maintenance of the strains in slant cultures. 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 µg l$^{-1}$) as that in the vitamin B mixture (Akasaka et al., 2004), and PYB$_{12}$ medium containing 10 g glucose l$^{-1}$ (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 was determined in PYB$_{12}$G medium supplemented with 0.1–20% (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 0.5–0.8 × 0.6–1.8 µm 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 µg 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 (3–6 µm) than those grown in PY4S agar and often formed irregular and

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Fig. 1. Phase-contrast photomicrographs of cells of strain WR061$^T$ grown on PY4S agar (a) and PYV4S agar (b). Bar, 5 µm.
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 OD600). 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 PY45 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 WR061T 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 WR061T and 1448 bp for strain WR054. There were two mismatches between the two sequences and the sequence of WR061T 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 canaliculitis, with a 16S rRNA gene sequence similarity of 91.6 % between P. propionicum JCM 5830T and the two novel strains. Propionibacterium avidum ATCC 25577T (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 5830T) 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 TechnoSurgura (Shimidu, Japan) (Moore et al., 1994). Both novel strains had almost the same fatty acid composition (Table 1). The major fatty acids of strains WR061T and WR054 were anteiso-C15:0 (~34.0 % for both strains), C15:0 dimethylacetal (DMA) (10 and 14.0 %, respectively) and anteiso-C15:0 DMA (6.7 and 9.2 %). The total percentages of C15:0 compounds in strains WR061T and WR054 were 72.6 and 70.0 %, respectively. Various DMA-type components were detected; the total amounts of DMA type components in strains WR061T 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 5830T, were anteiso-C15:0 (32.5 %), iso-C15:0 (31.0 %) and C19:0 cyclo DMA (ECL 19:322) (20.3 %). The presence of branched-chain C15:0 compounds as major components was common to the three strains; however, the overall profile of P. propionicum JCM 5830T 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).
Genomic DNA of strains WR061T, WR054 and P. propionicum JCM 5830T 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 mBondapak C18 column (3.9 × 300 mm; Waters). The genomic DNA G+C content of strains WR061T and WR054 were 69.5 and 69.3 mol%, respectively, and that of P. propionicum JCM 5830T 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 WR061T, WR054 and P. propionicum JCM 5830T 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 5830T was LL-DAP (consistent with that reported for this species; Schaal, 1986).

Some differential cellular and physiological characteristics of P. propionicum JCM 5830T, including cell morphology, cobalamin requirement and substrate utilization, were determined under the same conditions used for the characterization of strains WR061T and WR054. P. propionicum JCM 5830T 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 WR061T and WR054. P. propionicum JCM 5830T 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 5830T was also negative for catalase activity, whereas strains WR061T and WR054 were catalase-positive. Differences in other characteristics including substrate utilization are shown in Table 2.

Table 1. Cellular fatty acid composition (%) of strains WR061T and WR054 and their closest relative, Propionibacterium propionicum JCM 5830T.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>iso-C14:0</td>
<td>2.3</td>
<td>1.6</td>
<td>–</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.4</td>
<td>0.6</td>
<td>2.3</td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>7.5</td>
<td>8.6</td>
<td>31.0</td>
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<tr>
<td>anteiso-C15:0</td>
<td>33.7</td>
<td>34.6</td>
<td>32.5</td>
</tr>
<tr>
<td>C15:0</td>
<td>3.5</td>
<td>4.2</td>
<td>9.2</td>
</tr>
<tr>
<td>iso-C15:0 DMA</td>
<td>4.7</td>
<td>5.6</td>
<td>–</td>
</tr>
<tr>
<td>anteiso-C15:0DMA</td>
<td>9.2</td>
<td>6.7</td>
<td>–</td>
</tr>
<tr>
<td>C15:0 DMA</td>
<td>14.0</td>
<td>10.3</td>
<td>–</td>
</tr>
<tr>
<td>C16:0</td>
<td>0.4</td>
<td>1.6</td>
<td>2.3</td>
</tr>
<tr>
<td>C16:0 DMA</td>
<td>3.2</td>
<td>5.0</td>
<td>–</td>
</tr>
<tr>
<td>anteiso-C17:0</td>
<td>0.5</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>C17:0 DMA</td>
<td>7.2</td>
<td>4.2</td>
<td>–</td>
</tr>
<tr>
<td>C19:0 cyclo 9,10 DMA*</td>
<td>–</td>
<td>–</td>
<td>20.3</td>
</tr>
</tbody>
</table>

*Equivalent chain-length = 19.322.
**Description of Propioniciclava gen. nov.**

Propioniciclava (Pro.pi.on.i.ci.la.va. N.L. n. acidum pro-pionicum 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. Chemoorganotrophic. 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’də 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, malose, 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-C₁₅ : ₀ and C₁₅ : ₀ DMA are the major cellular fatty acids; C₁₅ : ₀ and DMA type compounds are the major fatty acid constituents.

The type strain, WR061ᵀ (＝JCM 15804ᵀ =DSM 22130ᵀ), 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 %.

**Acknowledgements**

This work was partly supported by a Grant-in-Aid from the Institute for Fermentation, Osaka, and also by the Project for Development of Technology for Analysing and Controlling the Mechanism of Biodegrading and Processing supported by the New Energy and Industrial Technology Development of Organization (NEDO). We are grateful to Y. Ohtaki for helpful assistance in the characterization of the strains.

**References**


