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

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Running title: *Propioniciclava tarda* gen. nov., sp. nov.

Abbreviations: CFA, whole-cell fatty acid; DMA, dimethylacetal; 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.

Facultatively anaerobic bacterial strains (WR061^{T}and WR054) were isolated from rice-straw residue in a methanogenic reactor treating waste from cattle farms in Japan. The two strains had almost the same phylogenetic and phenotypic properties. Cells were Gram-staining-positive, non-motile, non-spore-forming, irregular rods. Cobalamin (vitamin B_{12}) 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. The temperature range for growth was 20-37^{o}C, the optimum being 35^{o}C. They grew at pH 5.3-8.0, having a pH optimum at 6.8-7.5. Catalase and nitrate-reducing activities were detected. Aesculin was hydrolyzed. The major cellular fatty acids were anteiso-$C_{15:0}$ and $C_{15:0}$ DMA. Major respiratory quinone was menaquinone MK-9(H_4) and genome DNA G +

C content was 69.3-69.5%. Diagnostic diamino acid in the peptidoglycan was *meso*-DAP. Phylogenetic analysis based on the 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* was the most closely related species with sequence similarity of 91.6%. Based on the phylogenetic, physiological and chemotaxonomic analyses of the novel strains, *Propioniciclava tarda* gen. nov., sp. nov. is proposed to accommodate the strains. The type strain of the novel species is WR061^T (= JCM 15804^T = DSM 22130^T).

Propionate is one of major volatile fatty acids produced by fermentative bacteria during anaerobic decomposition of organic matter in methanogenic ecosystems. It often accumulates in methanogenic reactors to cause pH decrease and instability of the systems (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 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 (up to 1000 cattle in total) in Betsukai-machi in Hokkaido, Japan (Nishiyama *et al*., 2009a,b; Ueki *et al*., 2008, 2010). The reactor was a vertically-cylindrical type (1500 m^3) operated at 35^{o}C. Rice straw used as matting at the cattle farms, containing cattle faeces and urine, was 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 (10000 r.p.m.

for 10 min.) under N_2 atmosphere. The homogenized samples were successively diluted (10-fold) anaerobically and used as inocula to the anaerobic roll-tubes using PY4S medium containing a B-vitamin mixture (PYV4S) (Akasaka *et al*., 2004). Colonies that formed on the agar were picked at random after incubation for two weeks at 30ºC 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. *Propionibacterium propionicum* JCM 5830^T was used as a reference type strain of the closest species of both strains.

Both strains were cultivated anaerobically at 30ºC unless otherwise stated using peptone/yeast extract (PY) medium (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 used for maintenance of the strains in agar slants. PY liquid medium supplemented with the B-vitamin 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 added at the same concentration (10 µg l^{-1}) as that in the B-vitamin 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 tests and chemotaxonomic analyses. Cells previously transferred at least twice in the medium containing the vitamin mixture or cobalamin were used as inocula for various cultivation experiments. The pH of the media was usually adjusted to pH 7.4-7.5 except for determination of pH range for growth, since the strains preferred slightly alkaliphilic conditions as shown below. Growth in liquid media was monitored by changes in OD_{660}. The reference type strain of the closest species (*Propionibacterium propionicum* JCM

5830^T) was cultivated under the same conditions using the same media adjusted at pH 7.0.

Growth of the strains under aerobic conditions was examined as described previously (Ueki *et al*., 2008). Spore formation was assessed by observing cells after Gram staining, and production of thermotolerant cells was examined by cultivating heat-treated (80ºC for 10 min) cells in $PYB_{12}G$ liquid medium. The motility of cells (from both slant and liquid cultures) was examined using phase-contrast microscopy. Oxidase and nitrate-reducing activities were determined according to the methods described by Akasaka *et al*. (2003). Catalase activity was examined using cells cultivated in liquid media and collected by centrifugation. A small amount of H_2O_2 solution (3%, v/v) was mixed with the cell pellet (Wilkins *et al*., 1978). The optimum growth conditions were tested on temperatures at 10-45ºC (at 5ºC intervals with an exception at 37ºC), at pH 4.0, 5.3, 5.8, 6.8, 7.5, 8.0, 9.2 and 10.2 (as values verified after autoclaving), and in the presence of 0-5% (w/v) NaCl (at 1% intervals with an exception at 0.5%) in $PYB_{12}G$ medium. Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine] (Good's buffer; Dotite) (20mM) was used to adjusted the pH higher than 8.0 by using N_2 gas as the headspace (Ueki *et al*., 2008). Growth in the 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 30 mM as final concentrations. Utilization of each substrate was determined from growth measurement (OD_{660}) (except xylan and filter paper) as well as by determining fermentation products in the medium after cultivation. Fermentation products were analyzed by GC as described previously (Ueki *et al*., 1986). Production of urease, H_2S 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% (w/v) Oxgall (Difco). All experiments for characterization were performed in duplicate.

Strains WR061^T and WR054 grew poorly as colonies with slightly dry surface on PY4S agar slants. Cells were Gram-staining-positive and non-motile. Cells grown on PY4S agar were irregular and wedge-shaped rods, and usually occurred in pairs or chains with irregular V-shaped cell arrangements (Fig. 1A). Sizes of cells were 0.5-0.8 μm wide and 0.6-1.8 μm long. Spore formation was not observed and cells after heat treatment did not grow. The strains grew in air on either PY4S or nutrient agar. Cell morphology grown in the aerobic conditions was the same as those grown in the anaerobic conditions.

Growth of both strains in PY or PYG liquid medium was scanty, and the addition of B-vitamin mixture (PYVG) remarkably stimulated the growth. Cobalamin showed almost the same effect on the growth, indicating that both strains required cobalamin for growth. Thus, physiological characteristics of the strains were usually determined in the presence of cobalamin (PYB_{12} medium). The 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 the growth. When cells grown on PY4S agar slants were directly inoculated to $PYB_{12}G$ liquid medium, about 12 days were necessary to reach the early stationary phase, while the lag time was shortened to 3-4 days when cells cultivated on PYV4S slants were used for inoculation. Cells grown in PYV4S agar were slender and longer (3-6 μm) than those grown in PY4S agar, and usually observed as rather irregular and crescent–shaped arrangements. Mycelial forms were not observed (Fig. 1B).

The temperature range for growth was 20-37ºC; very weak growth at 37ºC and the optimum being 35ºC. The strains grew pH 5.3-8.0. 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 the measurement of OD_{660}). The final pH values in these media were 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); 0.5% (w/v) NaCl strongly suppressed the growth. Aesculin was hydrolyzed. Catalase activity was detected in cells grown in PY4S agar slants or in $PYB_{12}G$ medium. Nitrate-reducing activity was detected. The strains did not tolerate bile even at 0.1% (w/v) of Oxgall. Other physiological characteristics are shown in the species description.

The strains utilized fructose, galactose, glucose, cellobiose, lactose and salicin. The strains also utilized mannose, maltose, melibiose, saccharose, trehalose, melezitose, raffinose, mannitole, amygdalin, aesculin, pyruvate and lactate, however, the growth was delayed as compared with the substrates shown above. The strains did not utilize pentoses, polysaccharides and sugar alcohols. 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 PYB_{12} medium. The strain 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 $PYB_{12}G$ medium. Almost the same amounts of products were formed from other substrate utilized (the molar ratio of acetate and propionate is about 1:2). When grown on lactate (30 mM), 14.3 mmol l^{-1} of acetate and 34.4 mmol l^{-1} of propionate 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) as well as the methanogenic reactor (Ueki *et al*., 2008, 2010). Cobalamin is essential for methylmalonyl-CoA isomerase to catalyze propionate production in the methylmalonyl-CoA pathway (Roth *et al*., 1996).

The almost-complete 16S rRNA gene sequences were PCR amplified using the primer pair (8f and 1546r) and DNA samples extracted from cells 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 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 constructed with the neighbor-joining method (Saitou & Nei, 1987) by using the CLUSTAL W program (Thompson *et al*., 1994) and with the maximum-likelihood program (DnaML) of the PHYLIP 3.66 package (Felsenstein, 2006). All gaps and unidentified base positions in the alignments were excluded before sequence assembly. The sequence length determined was 1453 bp for strain WR061^{T} and 1448 bp for strain WR054. Both sequences had two mismatches and one gap (for WR061^{T}) (sequence similarity between the two strains, 99.86%). Phylogenetic analysis based on the 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 most closely related species of both strains was *Propionibacterium propionicum* (Charfrettag, *et al*., 1988; Cummains & Johnson, 1986; Gerencser & Slack, 1967; Schaal, 1986) isolated from human lacmal canaliculity with a 16S

rRNA gene sequence similarity of 91.6%. *Propionibacterium avidum* (Cummains & Johnson, 1986) was the next closely related species (sequence similarity, 91.2%). Both novel strains formed a distinct branch in the phylogenetic tree constructed by using the neighbour-joining method (Fig. 2). The tree topology evaluated by the maximum-likelihood method was essentially the same as that obtained with the neighbour-joining method (data not shown).

The two novel strains and the type strain of the closest relative (*Propionibacterium propionicum* JCM 5830^T) were cultivated under the same condition. Their cellular fatty acids (CFAs) were converted to methyl esters according to the method of Miller (1982) and analyzed by GC (Hp6890; Hewlett-Packard or G-3000; Hitachi) equipped with a HP Ultra 2 column. CFAs were identified from equivalent chain-lengths (ECL) (Miyagawa *et al*., 1979) according to the protocol of TechnoSuruga Co., Ltd (Shimidu, Japan) (Moore *et al*., 1994). Both novel strains had almost the same CFAs composition (Table 1). The major CFAs of the strains were anteiso-$C_{15:0}$ (about 34% for both strains), $C_{15:0}$ dimethylacetal (DMA) (10 and 14.0%) and anteiso-$C_{15:0}$ DMA (6.7 and 9.2%). The total percentages of $C_{15:0}$ compounds reached to 72.6% (strain WR061^T) and 70.0% (WR054), respectively. Various DMA type components were detected, and the total amounts of DMAs were 38.3% (WR061^T) and 31.8% (WR054), respectively. Unsaturated fatty acids were not detected. The major CFAs of the closest relative (JCM 5830^T) were anteiso-$C_{15:0}$ (32.5%), iso-$C_{15:0}$ (31%) 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 strain JCM 5830^T was significantly different from those of the novel strains. The presence of cyclo-type CFAs has not been reported for *Propionibacterium propionicum* (Charfreitag *et al*., 1988; Cummins & Moss, 1990; Kusano *et*

al., 1997; Schaal, 1986).

Genomic DNA of the three strains (WR061^T, WR054 and 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). Their G + C contents were measured by HPLC (HITACHI L-7400) equipped with a μBondapak C18 column (3.9 × 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 *Propionibacterium propionicum* JCM 5830^T was 65.3 mol% (consistent with the species description of 63-65%, Schaal, 1986). Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analyzed by using a mass spectrometer (JMS-SX102A; JEOL). Menaquinone MK-9(H_4) was the major respiratory quinone of the novel strains, which was the same as that of *Propionibacterium propionicum* (Charfreitag *et al*., 1988). Composition of cell wall diagnostic diamino acid of the three strains was analyzed according to the method described by Akasaka *et al*. (2003). The amino acid of the novel two strains was *meso*-diaminopimelic acid (DAP), while that of *Propionibacterium propionicum* JCM 5830^T was LL-DAP (consistent with the species description, Schaal, 1986).

Some differential cellular and physiological characteristics (including cell morphology, cobalamin requirement, substrates utilization) of *Propionibacterium propionicum* JCM 5830^T were determined under the same conditions used for the characterization for strains WR061^T and WR054. Strain JCM 5830^T produced dense and rough colonies with grayish surface on PY4S agar slants, and the colonies were composed of aggregates of distinctly long and branched, filamentous cells as described in the species

description (Schaal, 1986). Such filamentous assemblages of cells never occurred in the cultures of strains WR061^T and WR054. Strain JCM 5830^T did not require cobalamin, and produced acetate and propionate (about 1:2) from glucose irrespective of the presence or absence of cobalamin. Strain JCM 5830^T did not have catalase activity. Differences in other characteristics including substrate utilization are shown in Table 2.

The significantly low similarity of 16S rRNA gene sequence with the recognized closest species clearly indicates that strains WR061^T and WR054 should be affiliated to a novel taxon. Thus, based on the distinct differences of the cellular, physiological and chemotaxonomic characteristics of the novel strains from the closest species, *Propioniciclava tarda* gen. nov., sp. nov. is proposed to accommodate the strains. The type strain of the novel species is WR061^T (= JCM 15804^T = DSM 22130^T).

Description of *Propioniciclava* gen. nov.

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

Facultatively anaerobic. Cells are Gram-staining positive, irregular rods. Non-motile and non-spore forming. Chemoorganotroph. Ferments various carbohydrates and produces acetate and propionate from glucose. Has *meso*-DAP as the diagnostic diamino acid in the peptidoglycan. Major respiratory quinone is menaquinone MK-9(H_4). Has high G + C content of genome DNA. Type species 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).

Has the following characteristics in addition to those described for the genus description. Cobalamin (vitamin B_{12}) is essentially required for growth. In the absence of cobalamin, cells are irregular rods with tapered ends (0.5-0.8 μm wide and 0.6-1.8 μm long). Cells occur singly or in short chains. In the presence of cobalamin, cells are slender and irregular rods (3-6 μm long) with crescent–shaped arrangements. Growth is very slow even in the presence of cobalamin. Grows in aerobic conditions. The temperature range for growth is 20-37°C, the optimum being 35°C. Grows pH 5.3-8.0 and prefers slightly alkaliphilic conditions (up to pH 7.5). Addition of NaCl to the medium inhibits growth. Catalase and nitrate-reducing activities are positive. Aesculin is hydrolyzed. Oxidase, indole and hydrogen sulfide are not produced. Starch is not hydrolyzed. Sensitive to bile. Utilizes fructose, galactose, glucose, cellobiose, lactose and salicin as preferable substrates. The final pH after growth with glucose is 4.8-4.9. Acetate and propionate are major products with a small amount of succinate. Mannose, maltose, melibiose, saccharose, trehalose, melezitose, raffinose, mannitole, amygdalin, aesculin, pyruvate and lactate are also utilized with slow growth. Does not utilize 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. The G + C content of genomic DNA is 69.3-69.5%. Has anteiso-$C_{15:0}$ and $C_{15:0}$ DMA as the major cellular fatty acids; $C_{15:0}$ compounds and DMA type are the major components. The type strain of the novel species, 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. A reference strain (WR054 =

JCM 15805) was also isolated from the same reactor.

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References

Akasaka, H., Ueki, A., Hanada, S., Kamagata, Y. & Ueki, K. (2003). *Propionicimonas paludicola* gen. nov., sp. nov., a novel facultatively anaerobic, Gram-positive, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil. *Int J Syst Evol Microbiol* **53**, 1991-1998.

Akasaka, H., Ueki, K. & Ueki, A. (2004). Effects of plant residue extract and cobalamin on growth and propionate production of *Propionicimonas paludicola* isolated from plant residue in irrigated rice field soil. *Microbs Environ* **19,** 112-119.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389-3402.

Charfreitag O., Collins, M. D. & Stackebrandt, E. (1988). Reclassification of *Arachnia propionica* as *Propionibacterium propionica* comb. nov. *Int J System Bacteriol* **38,** 354-357.

Cummins, C. S. & Johnson, J. L. (1986). Genus I. *Propionibacterium* Orla-Jensen 1909, 337. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp 1346-1353. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Willkins.

Cummins, C. S. & Moss, C. W. (1990). Fatty acid composition of *Propionibacterium propionicum* (*Arachnia propionica*). *Int J Syst Bacteriol* **40,** 307-308.

Dolfing, J. (1988). Acetogenesis. In *Biology of anaerobic microorganisms*. pp. 417-468. Edited by A. J. B. Zehnder. John Wiley & Sons.

Felsenstein, J. (2006). PHYLIP (phylogeny inference package), version 3.66. Department of Genome Sciences, University of Washington, Seattle, USA.

Garrity, G. M. & Holt, J. G. (2001). The road map to the manual. In *Bergey's Manual of Systematic Bacteriology Second Edition*, Vol. 1, pp. 119-166. Edited by D. R. Boone & G. M. Garrity. NY: Springer.

Gerencser M. A. & Slack, J. M. 1(1967). Isolation and characterization of *Actinomyces propionicus*. *J Bacteriol* **94,** 109-115.

Holdeman, L. V., Cato, E. P. & Moore, W. E. C. (1977). *Anaerobe Laboratory Manual*, 4th edn. Blacksburg,

VA: Virginia Polytechnic Institute and State University.

Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19,** 161-207.

Kusano, K., Yamada, H., Niwa, M. & Yamasato, K. (1997). *Propionibacterium cyclohexanicum* sp. nov., a new acid-tolerant ω–cyclohexal fatty acid-containing *Propionibacterium* isolated from spoiled orange juice. *Int J Syst Bacteriol* **47**, 825-831.

Miller, L. T. (1982). Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxyl acids. *J Clin Microbiol* **16**, 584-586.

Miyagawa, E., Azuma, R. & Suto, E. (1979). Cellular fatty acid composition in Gram-negative obligately anaerobic rods. *J Gen Appl Microbiol* **25**, 41-51.

Moore, L. V. H., Bourne, D. M. & Moore, W. E. C. (1994). Comparative distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic Gram-negative bacilli. *Int J Syst Bacteriol* **44**, 338-347.

Nishiyama, T., Ueki, A., Kaku, N. & Ueki, K. (2009a). *Clostridium sufflavum* sp. nov., isolated from a methanogenic reactor treating cattle waste. *Int J Syst Evol Microbiol* **59**, 981-986.

Nishiyama, T., Ueki, A., Kaku, N. Watanabe , K. & Ueki, K. (2009b). *Bacteroides graminisolvens* sp. nov., a xylanolytic anaerobe isolated from a methanogenic reactor treating cattle waste. *Int J Syst Evol Microbiol* **59**, 1901-1907.

Roth, J. R., Lawrence, J. G. & Bobik, T. A. (1996). Cobalamin (coenzyme B_{12}): synthesis and biological significance. *Annu Rev Microbiol* **50,** 137-181.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-425.

Schaal, K. P. (1986). Genus *Arachnia* Pine and Georg 1969, 269^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp 1332-1342. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Willkins.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680.

Ueki, A., Matsuda, K. & Ohtsuki, C. (1986). Sulfate reduction in the anaerobic digestion of animal waste. *J Gen Appl Microbiol* **32**, 111-123.

Ueki, A., Abe, K., Kaku, N., Watanabe, K. & Ueki, K. (2008). *Bacteroides propionicifaciens* sp. nov.,

isolated from rice-straw residue in a methanogenic reactor treating waste from cattle farms. *Int J Syst Evol Microbiol* **58**, 346-352.

Ueki, A., Abe, K., Ohtaki, Y., Kaku, N., Watanabe, K. & Ueki, K. (2010). *Bacteroides paurosaccharolyticus* sp. nov., isolated from a methanogenic reactor treating waste from cattle farms. *Int J Syst Evol Microbiol* **60**, in press.

Wilkins, T. D., Wagner, D. L., Veltri, B. J. Jr. & Gregory, E. M. (1978). Factors affecting production of catalase by *Bacteroides*. *J Clin Microbiol* **8**, 553-557.

Figure legends

Fig. 1. Phase-contrast photomicrographs of cells of strain WR061^T: A, grown on PY4S agar; B, grown on PYV4S agar. Bar, 5 μm.

Fig. 2. Neighbor-joining tree showing the phylogenetic relationship of novel strains (WR061^T and WR054) and other species in the family *Propionibacteriaceae* as well as some representative type species of other families in the order *Actinomycetales* based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) above 70% are shown at branch nodes. The sequence of *Acidimicrobium ferroxidans* JCM 1546^T was used as the outgroup. The tree topology evaluated by using the maximum-likelihood method was almost the same as that obtained with the neighbour-joining method. Bar, 1% estimated difference in nucleotide sequence position.

Fig. 1

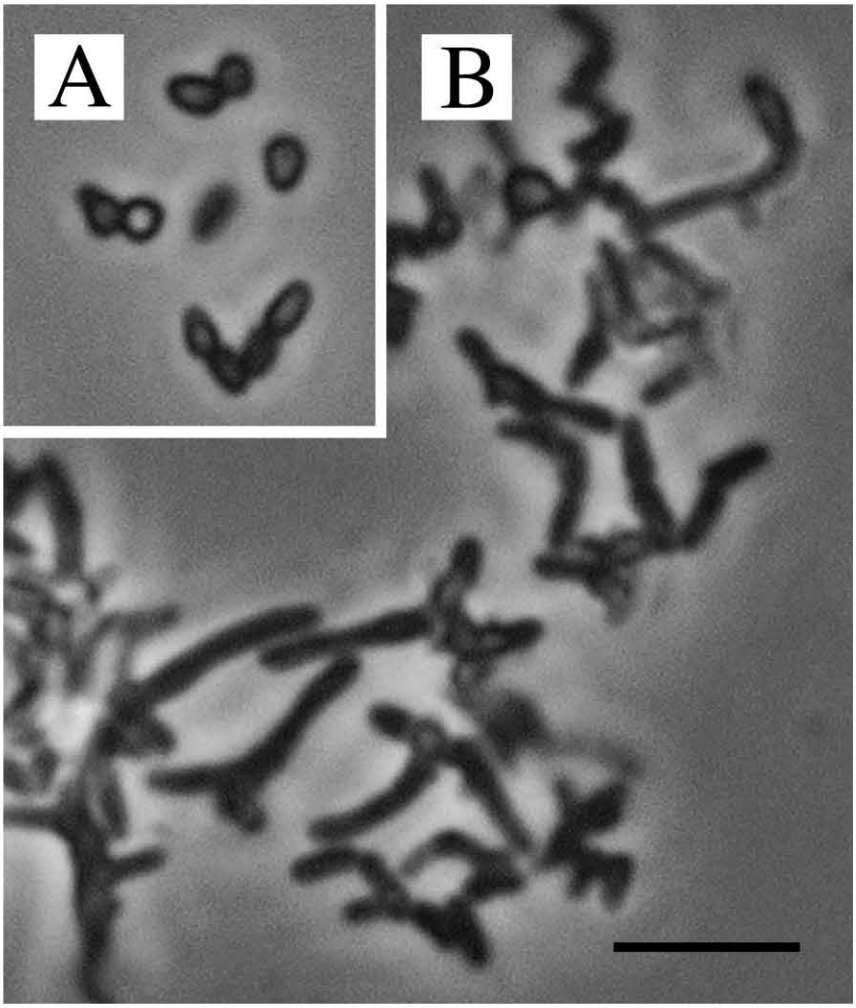

Fig. 2

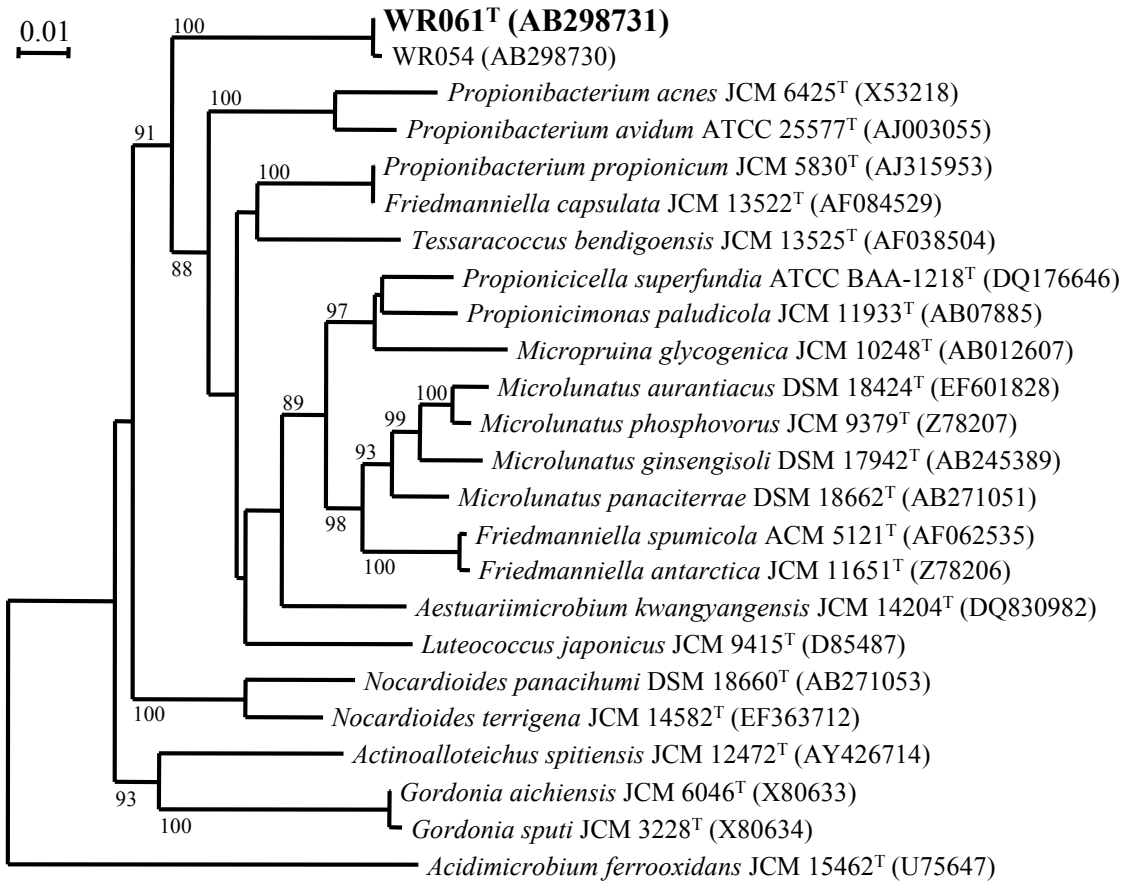
0.01
WR061T (AB298731)
WR054 (AB298730)
Propionibacterium acnes JCM 6425T (X53218)
Propionibacterium avidum ATCC 25577T (AJ003055)
Propionibacterium propionicum JCM 5830T (AJ315953)
Friedmanniella capsulata JCM 13522T (AF084529)
Tessaracoccus bendigoensis JCM 13525T (AF038504)
Propionicicella superfundia ATCC BAA-1218T (DQ176646)
Propionicimonas paludicola JCM 11933T (AB07885)
Micropruina glycogenica JCM 10248T (AB012607)
Microlunatus aurantiacus DSM 18424T (EF601828)
Microlunatus phosphovorus JCM 9379T (Z78207)
Microlunatus ginsengisoli DSM 17942T (AB245389)
Microlunatus panaciterrae DSM 18662T (AB271051)
Friedmanniella spumicola ACM 5121T (AF062535)
Friedmanniella antarctica JCM 11651T (Z78206)
Aestuariimicrobium kwangyangensis JCM 14204T (DQ830982)
Luteococcus japonicus JCM 9415T (D85487)
Nocardioides panacihumi DSM 18660T (AB271053)
Nocardioides terrigena JCM 14582T (EF363712)
Actinoalloteichus spitiensis JCM 12472T (AY426714)
Gordonia aichiensis JCM 6046T (X80633)
Gordonia sputi JCM 3228T (X80634)
Acidimicrobium ferrooxidans JCM 15462T (U75647)
100
100
91
100
88
97
89
100
99
93
98
100
100
93
100

Table 1. Cellular fatty acid composition (%) of strains WR061^T, WR054 and the closest relative.

Strains: 1, WR061^T; 2, WR054; 3, *Propionibacterium propionicum* JCM 5830^T.
-, Not detected; DMA, dimethylacetal; cyclo, cyclopropan fatty acid.
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	-
$C_{19:0}$ cyclo9,10 DMA*	-	-	20.3

* Equivalent chain length = 19.322.

Table 2. Some differential characteristics of strains WR061^{T}and WR054 from the closest relative . 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.

Characteristic	1	2	3
Habitats	Methanogenic reactor	Methanogenic reactor	Human
Cell morphology	Irregular and slender rods	Irreugular and slender rods	Pleomorphic, filamentous rods
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+	+
G + C content (mol%)	69.5	69.3	65.3
Diamino acid	*meso* -DAP	*meso* -DAP	*LL* -DAP
CFA	anteiso-$C_{15:0}$, $C_{15:0}$ DMA, anteiso-$C_{15:0}$ DMA	anteiso-$C_{15:0}$, $C_{15:0}$ DMA, anteiso-$C_{15:0}$ DMA	anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{19:0}$ cyclo 9,10