Falcatimonas natans gen. nov., sp. nov., a strictly anaerobic, amino-acid-decomposing bacterium isolated from a methanogenic reactor of cattle waste

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A strictly anaerobic bacterial strain (WN011\(^T\)) was isolated from a methanogenic reactor treating waste from cattle farms. Cells of the strain were Gram-stain-negative curved rods with a polar flagellum. Spores were not produced. The optimum temperature for growth was 35–37 °C and the optimum pH was 6.7. The strain did not utilize carbohydrates as growth substrates. The strain grew in PY medium and produced acetate, butyrate, isovalerate and \(\mathrm{H}_2\) as well as propionate and isobutyrate as minor products. Amino acids (L-isoleucine, L-leucine, L-lysine, L-serine, L-threonine and L-valine) added to PY medium enhanced growth of the strain and increased the amounts of fermentation products. Oxidase, catalase and nitrate-reducing activities were negative. Hydrogen sulfide was produced. The genomic DNA G+C content was 38.8 mol %.

Compounds related to iso-C\(_{15}:0\) (fatty acid, dimethylacetal and aldehyde) were detected as predominant components by the cellular fatty acids analysis. The diagnostic diamino acid of the cell-wall peptidoglycan was meso-diaminopimelic acid. On the basis of 16S rRNA gene sequences, three clones from wastewater were very closely related to strain WN011\(^T\) (up to 99.9 % sequence similarity). The most closely related described species were those in cluster XIVa of the class Clostridia such as Ruminococcus gauvreauii (93.8 % 16S rRNA gene sequence similarity), Clostridium fimetarium (93.5 %) and Clostridium bolteae (93.5 %). Based on the distinct differences in phylogenetic and phenotypic characteristics of strain WN011\(^T\) from those of related species, it is concluded that strain WN011\(^T\) represents a novel species of a new genus in the family Lachnospiraceae, for which the name Falcatimonas natans gen. nov., sp. nov. is proposed. The type strain of the type species is WN011\(^T\) (=JCM 16476\(^T\)=DSM 22923\(^T\)).

Decomposition of protein is one of the major processes in methanogenesis from waste containing a wide variety of organic compounds like municipal sewage sludge and dairy waste. During the course of anaerobic degradation of organic matter, protein is hydrolysed by proteolytic microbes, and amino acids generated are fermented by aminolytic microbes to mainly volatile fatty acids (VFAs) that include acetate, propionate, butyrate and branched-chain fatty acids (isobutyrate and isovalerate) as well as \(\mathrm{H}_2\) and \(\mathrm{CO}_2\) (Holdeman et al., 1977). Each amino acid is converted to different kinds of VFAs depending on the combinations of amino acids and bacterial species concerned (Buckel, 1999; Ramsay & Pullammanappallil, 2001; Smith & Macfarlane, 1997). Thus, degradation of protein and amino acids is one of the key reactions for efficient methanogenesis in anaerobic treatment of waste containing various organic compounds. Many members within the order Clostridiales of the phylum Firmicutes are asaccharolytic and decompose protein or amino acids in anaerobic circumstances (Rainey et al., 2009). We have recently described two species of amino-acid-decomposing anaerobic bacteria, Anaerosphaera aminiphila and Aminicella lysinilytica (name corrected from Aminocella lynsionlytica on validation), that were both isolated from the same methanogenic reactor. Anaerosphaera aminiphila, phylogenetically placed within the family Peptostreptococcaceae (formerly clostridia cluster XIII), utilized L-glutamate, L-glutamine, L-histidine and L-arginine and produced acetate and butyrate (Ueki et al.,

Abbreviations: CFA, cellular fatty acid; DMA, dimethylacetal; VFA, volatile fatty acid.

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain WN011\(^T\) is AB298726.

One supplementary figure and two supplementary tables are available with the online Supplementary Material.
Aminicella lysinilytica, in the family Eubacteriaceae, fermented L-lysine as well as L-arginine to acetate and butyrate in the presence of vitamin B$_{12}$ as an essential growth factor (Oren & Garrity, 2015; Ueki et al., 2015). In the present study, another strictly anaerobic, amino-acid-decomposing bacterium (strain WN011$^T$) also isolated from the same reactor is described. The strain was asaccharolytic and fermented peptone as well as several kinds of amino acids to mainly acetate, butyrate, isovalerate and H$_2$. The most closely related described species of the strain were those in clostridia cluster XIVa with 16S rRNA gene sequence similarities of 93–94%. Clostridial 16S rRNA cluster XIVa or the family Lachnospiraceae comprises phenotypically diverse, anaerobic bacterial species that differ in Gram-staining, cell morphology, spore formation, motility, fermentation products, etc. (Liu et al., 2008; Rainey et al., 2009). Cells of strain WN011$^T$ were Gram-stain-negative, non-spore-forming, motile and crescent-shaped rods. Since most members in clostridial cluster XIVa are asaccharolytic, the asaccharolytic and aminolytic properties of strain WN011$^T$ expand the phenotypic diversity of the species in the group. According to the proposal for restriction of the genus Clostridium to the species in clostridia cluster I (Clostridium sensu stricto; Clostridium butyricum and related species) (Lawson & Rainey, 2016; Rainey et al., 2009), a novel species of a new genus in the family Lachnospiraceae is proposed to accommodate the strain. The novel species added a further insight concerning involvement of various kinds of bacterial species in anaerobic degradation of compounds derived from protein.

Strain WN011$^T$ was isolated from a methanogenic reactor treating waste collected from cattle farms (comprising up to 1000 cattle in total) in Betsukai-machi, Hokkaido, Japan (Abe et al., 2012; Ueki et al., 2016). Strain WN011$^T$ was recovered from a rice-straw residue sample obtained from the reactor as described previously (Sugawara et al., 2011; Ueki et al., 2011).

Strain WN011$^T$ was cultivated anaerobically at 30 °C unless otherwise stated by using peptone/yeast extract (PY) as basal medium with oxygen-free mixed gas (95% N$_2$/5% CO$_2$) as the headspace. PY medium contained (l$^{-1}$) 10 g trypticase (BBL), 5 g peptone, 0.2 g Na$_2$CO$_3$, 0.3 g L-cysteine-HCl·2H$_2$O (as a reducing agent) and 1 mg resazurin-Na (as a redox indicator) as well as salt solutions (Satoh et al., 2002). PY medium supplemented with L-$\alpha$-200.25 g each of glucose, cellobiose, maltose and soluble starch together with 15 g agar was designated PY4S agar and used for isolation and maintenance of the strain in agar slants. All media were usually adjusted to pH 7.2–7.4 (pH 6.8–7.0 after autoclaving) with 1 M NaOH.

Growth of the strain under aerobic conditions was examined as described previously (Ueki et al., 2011). The motility of cells was examined by phase-contrast microscopy and flagella staining was carried out according to Blenden & Goldberg (1965). Spore formation was assessed by phase-contrast microscopy and observation of cells after Gram-staining. Furthermore, to test spore production and the presence of thermostolerant cells, cultures grown in PYG medium or on agar slants were inoculated to fresh PYG medium and the culture tubes were treated at 80 °C for 10 min before cultivation at 30 °C.

Cells of strain WN011$^T$ were small curved or crescent-shaped rods with tapered ends; most cells were approximately 0.4–0.6 μm in diameter and 1.3–2.3 μm in length. Cells usually occurred singly (Fig. S1a, available in the online Supplementary Material). The cells were Gram-stain-negative and motile by tumbling as observed by phase-contrast microscopy. Flagella staining showed cells had a single polar flagellum (Fig. S1b). Surface colonies on PY4S agar were 0.5–1.0 mm in diameter after 48 h of anaerobic incubation, circular, thin, smooth, greyish and translucent. Cells of the strain did not grow in air. Spore formation was not observed by microscopy and heat-tolerant cells were not detected.

Utilization of carbon sources was tested in PY liquid medium with each substrate (monosaccharides, disaccharides, oligosaccharides and sugar alcohols) added at 10 g l$^{-1}$. Other substrates (polysaccharides, glycosides and Casamino acids) were added at 5 g l$^{-1}$. To determine amino acids utilization, both PY medium and 1/10 PY medium (concentrations of both tryptase and yeast extract were decreased to one tenth of those in PY medium) were used as basal media. The final concentration of each amino acid added was 30 mM. Utilization of each substrate was determined by growth monitored by OD$_{660}$ as well as by measurement of fermentation products after cultivation. Fermentation products (VFAs, H$_2$ and CO$_2$) were analysed by GC and the amounts of products in the presence of substrates were compared with those in the basal media without added substrates. All tests were carried out in duplicate and reproducibility was checked. Strain WN011$^T$ grew rapidly in PY medium without additional substrates. After 48 h in PY medium, the strain produced (as mean values of amounts obtained from different experiments of cultivation): acetate (10.9 mmol l$^{-1}$), butyrate (15.5 mmol l$^{-1}$), isovalerate (8.2 mmol l$^{-1}$) and H$_2$ (4.9 mmol l$^{-1}$) as well as CO$_2$ as major products, together with propionate (0.4 mmol l$^{-1}$) and isobutyrate (2.3 mmol l$^{-1}$) as minor products. Carbohydrates including glucose did not improve the growth and production of VFAs (Table S1). Addition of six amino acids (L-isoleucine, L-lysine, L-serine, L-threonine and L-valine) individually to PY medium promoted growth and production of VFAs in each case. The concentration of acetate was increased by the addition of L-serine, whereas production of both acetate and butyrate was enhanced with L-lysine or L-threonine. Addition of branched-chain amino acids (L-isoleucine, L-lysine and L-valine) raised production of branched-chain VFAs (isovalerate with L-isoleucine or L-lysine; isobutyrate with L-valine). Other amino acids did not affect growth and fermentation products. Casamino acids slightly enhanced production of isovalerate. Only low amounts of VFAs were produced in 1/10 PY basal medium and thus increases in the concentrations of VFAs in the
presence of each amino acid were clearly shown (Table S1). The results for amino acids utilization obtained by using the two basal media matched well. Final pH after growth in PY medium with or without amino acids utilized was 6.2–6.7. We did not determine the concentration of ammonium in the medium after cultivation, but ammonium generated by deamination of amino acids would act to suppress pH decrease caused by increase in VFAs concentrations in the medium. Good growth and production of various VFAs in PY medium without additional substrates indicated that proteinous compounds in the medium composed of amino acids shown above must support growth of the strain. B-vitamin requirement was not recognized. The substrates tested but not utilized are shown in the species description.

The optimum growth conditions of strain WN011T were tested in PYG medium at temperatures of 10–45 °C (at 5 °C intervals with an exception at 37 °C), at pH4.1, 4.9, 5.8, 6.7, 7.5, 8.3 and 10.1 (as values verified after autoclaving), and by the addition of 0–50 g l⁻¹ NaCl (at 10 g l⁻¹ intervals with exceptions of 3 and 6 g l⁻¹) in the medium. The temperature range for growth was 15–40 °C, with the highest growth rate at 35–37 °C. The strain grew at pH 5.8–7.5, with optimum growth at pH 6.7. The strain grew in medium containing up to 20 g l⁻¹ NaCl; the highest growth rate was obtained without NaCl addition. The specific growth rate (μ) at the optimum condition (35 °C and pH 6.7 in the absence of added NaCl) was about 0.25 h⁻¹.

Other physiological characterization was carried out according to the methods described by Holdeman et al. (1977) and Ueki et al. (2011). Catalase, oxidase and nitrate-reducing activities were not detected. Strain WN011T produced hydrogen sulfide. Aesculin was hydrolysed. Other physiological characteristics are presented in the species description.

Cells cultivated at 30 °C until the stationary phase of growth (for 48 h) in PYG medium were used for following chemotaxonomic analyses. Genomic DNA was extracted from the cells according to the method described by Akasaka et al. (2003) and digested with P1 nuclease by using a YAMASA GC kit (Yamasa Shoyu), and the G+C content was measured by HPLC (L-7400; Hitachi) equipped with a µBondapack C18 column (3.9×300 mm; Waters). Whole-cell fatty acids (CFAs) were converted to methyl esters according to the method of Miller (1982) and analysed by GC equipped with a HP Ultra 2 column. CFAs were identified by equivalent chain-length at TechnoSuruga Co. (Shizuoka, Japan) (Moore et al., 1994). Composition of cell-wall diagnostic diamino acids was analysed according to the method described by Akasaka et al. (2003). Respiratory isoprenoid quinones were extracted and analysed according to the method described by Komagata & Suzuki (1987) for the presence of menaquinones and ubiquinones. The G+C content of the genomic DNA of strain WN011T was 38.8 mol%. The major components detected by the analysis of CFAs were iso-C₁₅:₀ dimethylacetal (DMA) (27.7%), iso-C₁₅:₀ aldehyde (23.7%) and iso-C₁₅:₀ (10.0%). Compounds related to C₁₄:₀, C₁₆:₀ and C₁₇:₀ were detected as minor components (Table S2). The cell-wall peptidoglycan contained meso-diaminopimelic acid as a diagnostic diamino acid. Neither menaquinones nor ubiquinones were detected.

DNA was extracted from cells as described previously (Akasaka et al., 2003). The almost full-length 16S rRNA gene was amplified by PCR using a primer set of 27f and 1546r (Brosius et al., 1981). The PCR-amplified 16S rRNA gene was 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 sequences with reference sequences in the GenBank database were performed with the BLAST program (Altschul et al., 1997). A phylogenetic tree was reconstructed with the neighbour-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, 2004). All gaps and unidentified base positions in the alignments were excluded before sequence assembly.

Analysis of the almost-complete 16S rRNA gene sequence (1504 bp) of strain WN011T assigned the strain to the phylum Firmicutes. Two clones (GenBank accession numbers HM445978 and HM445979) from the same wastewater sample were placed as the closest relatives (99.9 and 99.8% 16S rRNA gene sequence similarity, respectively) based on pairwise comparison of sequences. Another environmental clone (GenBank accession no. CR933122) derived from an anaerobic sludge digester (Chouari et al., 2005) was the next closest relative with a sequence similarity of 98.9%. The most closely related described species of strain WN011T were members in clostridial 16S rRNA cluster XIVa (Collins et al., 1994; Liu et al., 2008; Rainey et al., 2009) with similar levels of sequence similarities: Ruminococcus gauvreauii JCM 14987T (Domingo et al., 2008) (16S rRNA gene sequence similarity of 93.8%), Clostridium firmi- tarium DSM 9179T (93.5%) (Rainey et al., 2009), Clostridium bolteae ATCC BAA-613T (Song et al., 2003) (93.5%), Clostridium oroticum ATCC 13619T (93.4%) (Rainey et al., 2009), Agathobacter rectalis (=Eubacterium rectale) ATCC 33656T (93.1%) (Rosero et al., 2016; Wade, 2009) and Clostridium saccharolyticum ATCC 35040T (93.1%) (Rainey et al., 2009). The phylogenetic tree comprised of these relatives showed that strain WN011T and the three closely related environmental clones formed a distinct cluster from any of the related species. All six related recognized species listed above were affiliated with different clusters and strain WN011T was placed equally distant from any of these species (Fig. 1).

Some characteristics of strain WN011T were compared with the related species as representatives of the neighbouring clusters shown in the phylogenetic tree. The characteristics of cells of strain WN011T (Gram-stain-negative, non-spore-forming, curved rods) are unique among the related species, since most of them are Gram-stain-positive rods or oval to cocoid cells. Strain WN011T is asaccharolytic, while all
other relatives shown above are carbohydrate-utilizers. All related species do not produce branched-chain VFAs. The CFA composition of strain WN011T was fundamentally different from those of related species. Compounds related to odd-numbered C\textsubscript{15-0} branched-chain fatty acid (including DMA and aldehyde) were the major compounds for strain WN011\textsuperscript{T}, while even-numbered compounds such as C\textsubscript{14-0}, C\textsubscript{16-0}, C\textsubscript{16-1} DMA and C\textsubscript{18-1} DMA were the major components for the related species (Table 1).

Detection of almost identical sequences in wastewater and anaerobic sludge from different geographical locations (China and France) confirmed a wide distribution of the species were distinctly different from those of strain WN011\textsuperscript{T}. The sequence of Clostridium colinum DSM 6011\textsuperscript{T} (a species in clostridial cluster XIVb) (Rainey et al., 2009) was used as the outgroup. Bar, 2% estimated difference in nucleotide sequence.

**Fig. 1.** Neighbour-joining tree showing the phylogenetic relationship of strain WN011\textsuperscript{T} to representative species in clostridial cluster XIVa based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) >70 \% are shown at branch nodes. The sequence of Clostridium colinum DSM 6011\textsuperscript{T} (a species in clostridial cluster XIVb) (Rainey et al., 2009) was used as the outgroup. Bar, 2% estimated difference in nucleotide sequence.

Description of *Falcatimonas gen. nov.*

*Falcatimonas* (Fal.ca.ti.mo’nas L. adj. falcatus sickle- or crescent-shaped; Gr. n. monas a unit; N.L. fem. n. Falcatimonas a sickle-shaped unit).

Strictly anaerobic, motile rods. Gram-stain-negative. Chemoorganotrophic and fermentative. Does not utilize carbohydrates. Grows well in PY medium. Utilizes amino acids and produces volatile fatty acids. Oxidase and catalase activities are negative. Compounds related to iso-C\textsubscript{15-0} (fatty acid, DMA or aldehyde) are major components of cellular fatty acids. The cell-wall peptidoglycan contains meso-diaminopimelic acid as the diagnostic amino acid. Does not have any respiratory quinones. The genus belongs to the family Lachnospiraceae in the order Clostridiales.

The type species is *Falcatimonas natans*.

Description of *Falcatimonas natans* gen. nov., sp. nov.

*Falcatimonas natans* (na’tans L. adj. natans swimming, referring to the mobility of cells).
Cells are small and curved rods with tapered ends, 0.4–0.6 µm in diameter and 1.3–2.3 µm in length. Occurs singly. Motile with a polar flagellum. Spore formation is not observed and thermotolerant cells are absent. Colonies on PY4S agar are smooth surface. B-vitamins are not required for growth. The final pH after growth in PY medium is 6.2–6.7 irrespective of the presence of added amino acids. Nitrate-reducing activity is negative. Hydrogen sulfide is produced. Aesculin is hydrolysed. Indole, lecithinase and lipase are not produced. Milk is unchanged and no growth occurs in chopped meat broth. The predominant cellular fatty acids are iso-C<sub>15:0</sub>DMA, iso-C<sub>15:0</sub> aldehyde and iso-C<sub>15:0</sub>.

The type strain, WN011<sup>T</sup> (=JCM 14987<sup>T</sup>=DSM 22923<sup>T</sup>) was isolated from rice-straw residue in a methanogenic reactor treating waste from cattle farms in Japan. The genomic DNA G+C content of the type strain is 38.8 mol%.

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**References**


