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TAXONOMIC DESCRIPTION
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Aminipila butyrica gen. nov., sp. nov., a strictly anaerobic, arginine-decomposing bacterium isolated from a methanogenic reactor of cattle waste

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Abstract

A strictly anaerobic bacterial strain (FH042^T) was isolated from a methanogenic reactor treating waste from cattle farms. Cells were stained Gram-positive, straight to gently curved rods with polar flagella. The strain was asaccharolytic. The strain fermented amino acids (L-arginine, L-lysine and L-serine) as growth substrates and produced acetate and butyrate. The optimum temperature for growth was 30 °C and the optimum pH was 6.1–6.8. Oxidase, catalase and nitrate-reducing activities were negative. Hydrogen sulfide was produced. The genomic DNA G+C content of strain FH042^T was 44.7±0.2 mol%. The major cellular fatty acids were $C_{18:1}\omega9c$ DMA, $C_{17:2}/C_{17:1}\omega9c$ (as summed feature), $C_{16:0}$ DMA and $C_{14:0}$. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid as a diagnostic amino acid. The most closely related described species on the basis of 16S rRNA gene sequences was *Anaerovorax odorimutans* in the family XIII *Incertae Sedis* in the order *Clostridiales* of the class *Clostridia* with sequence similarity of 95.1 %. Based on the distinct differences in phylogenetic and phenotypic characteristics between strain FH042^T and related species, *Aminipila butyrica* gen. nov., sp. nov. is proposed to accommodate the strain. Type strain is FH042^T (=JCM 31555^T=DSM 103574^T).

In anaerobic decomposition of organic matter, protein is hydrolysed to amino acids and then the amino acids are fermented to volatile fatty acids (VFAs) such as acetate, propionate, butyrate and branched fatty acids (isobutyrate and isovalerate) as well as H_2 and CO_2 by anaerobic bacterial populations. Each amino acid is converted to different combinations of VFAs depending on the amino acids fermented and bacterial species involved [1–3]. Investigation into ecology or physiology of protein- or amino acid-degrading anaerobic bacteria is important to understand the methanogenic process of wastewater treatment. Recent descriptions of novel anaerobic species of amino acid-degrading bacteria isolated from methanogenic reactors [4–10] indicate that many phylogenetically diverse species are involved in degradation of proteinous compounds or amino acids. In this study, a strictly anaerobic, Gram-positive, amino acid-degrading bacterium (strain FH042^T) isolated from a methanogenic reactor is described. The strain fermented L-arginine, L-lysine and L-serine and produced acetate and butyrate. Based on the analysis of 16S rRNA gene sequence, the strain was assigned to the class *Clostridia* in the phylum *Firmicutes* and affiliated with the family XIII *Incertae Sedis* in the order *Clostridiales* [11]. We propose *Aminipila butyrica* gen. nov., sp. nov. to accommodate the strain. Type strain is FH042^T (=JCM 31555^T=DSM 103574^T).

Strain FH042^T was isolated by the anaerobic roll-tube method for the colony-counting of anaerobic fermentative bacteria [12] from a methanogenic reactor treating waste collected from cattle farms (housing up to 1000 cattle in total) in Betsukai-machi, Hokkaido, Japan, as described previously [7–10]. Strain FH042^T was one of the isolates obtained from a digester fluid sample without plant residue or other solid material collected from the reactor. The pure culture of the strain was obtained after repeats of the colony isolation using the anaerobic roll-tube method. The purity of the strain was confirmed by observing colonies on the roll tube agar and the cell morphology under a phase-contrast microscope. The purity of the strain was checked routinely by observing cells microscopically as well as by measuring fermentation products after cultivation in PY or PY–arginine medium (see below). The strain was cultivated anaerobically at 30 °C unless otherwise stated by using peptone/yeast extract (PY) basal medium with oxygen-free mixed gas (95 % N_2/5 % CO_2) as the headspace as described

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Keywords: amino acid decomposition; anaerobes; *Clostridiales*; family XIII; methanogenic reactor.
Abbreviations: CFA, cellular fatty acid; DMA, dimethylacetal; ECL, equivalent chain length; ML, maximum-likelihood; NJ, neighbour-joining; VFA, volatile fatty acid.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain FH042^T is AB298771.
Two supplementary tables and two supplementary figures are available with the online version of this article.

previously [7–10]. PY medium contained (l^{-1}) 10 g Trypticase (BBL), 5 g yeast extract, 0.2 g Na_2CO_3, 0.3 g L-cysteine hydrochloride dihydrate (as a reducing agent) and 1 mg sodium resazurin (as a redox indicator) as well as salt solutions [12, 13]. PY medium supplemented with (l^{-1}) 0.25 g each of glucose, cellobiose, maltose and soluble starch together with 15 g agar was designated as PY4S agar and used for isolation and maintenance of the strain in agar slants. All media were usually adjusted to pH 7.1–7.2 (before autoclaving) with 1 M NaOH.

Growth of the strain under aerobic conditions was examined as described previously [7]. Spore formation was assessed by phase-contrast microscopy and observation of cells after Gram-staining. To examine formation of thermotolerant cells, cells cultivated in PY–arginine medium or on PY4S agar slants were inoculated to PY–arginine medium, and the culture tubes were treated at 80 °C for 10 min before cultivation at 30 °C. The motility of cells was examined by phase-contrast microscopy. Flagella staining was carried out according to Blenden and Goldberg [14].

Colonies of strain FH042^T were thin and translucent with a smooth surface on agar slants after 48 h of inoculation. Cells were Gram-stain-positive and straight to gently curved or sigmoid rods with flat ends, usually 0.7–1.0 µm in diameter and 3.0–8.0 µm in length. Longer rods up to 40 µm often occurred (Fig. S1a, available in the online version of this article). Cells were motile; one or two polar flagella were observed by flagella staining of cells (Fig. S1b–d). Cells of the strain did not grow in air. Spores were not observed in cells by microscopy, irrespective of the medium used for cultivation of the strain such as PY, PY–arginine and PY4S agar slants. The strain grew after heat-treatment, indicating the presence of thermotolerant cells.

Utilization of carbon sources was tested in PY liquid medium with monosaccharides, disaccharides, trisaccharides and sugar alcohols added at 10 g l^{-1}. Polysaccharides and glycosides were added at 5 g l^{-1}, and organic acids as well as amino acids were added at 30 mM as the final concentration in the medium. Utilization of each substrate was usually determined by growth measured by OD_{660} of culture as well as measurement of pH and fermentation products after cultivation. Fermentation products (fatty acids and gases) were analysed by gas chromatography [15]. All tests were carried out in duplicate.

Strain FH042^T grew in PY medium without additional substrates. The strain produced (as mean values of amounts obtained from different experiments of cultivation) acetate (7.3 mmol l^{-1}) and butyrate (3.3 mmol l^{-1}) after 72 h of cultivation in PY medium. Final pH after growth in PY medium was 6.6. Carbohydrates and organic acids (arabinose, ribose, xylose, fructose galactose, glucose, mannose, rhamnose, sorbose, cellobiose, lactose, maltose, melibiose, sucrose, trehalose, melezitose, raffinose, carboxymethylcellulose, cellulose, dextrin, glycogen, inulin, pectin, starch, xylan, dulcitol, inositol, mannitol, sorbitol, aesculin, amygdalin, salicin, glycerol, fumarate, lactate, malate, pyruvate and succinate) added to PY medium did not improve the growth and production of VFAs. Three kinds of amino acids (L-arginine, L-lysine and L-serine) enhanced the growth and production of VFAs; 12.7 mmol l^{-1} acetate and 12.7 mmol l^{-1} butyrate from L-arginine, 34.5 mmol l^{-1} acetate and 15.6 mmol l^{-1} butyrate from L-lysine, and 16.0 mmol l^{-1}acetate from L-serine (Table S1). A trace amount of H_2 (2.15 mmol l^{-1}) was produced from L-arginine in PY medium. The final pH values were pH 7.7 with L-arginine and pH 5.2 with L-lysine (Table S1). We did not determine the concentrations of ammonium in the medium after cultivation, but the balance of concentrations of VFAs and ammonium generated by deamination of amino acids would affect the pH values in the medium. Although concentrations of VFAs produced were higher in the medium with L-lysine, the highest growth (OD_{660}) was obtained in the medium containing L-arginine. Casamino acids moderately enhanced production of acetate (10.2 mmol l^{-1}) and butyrate (4.8 mmol l^{-1}). Other 14 kinds of proteinogenic amino acids (L-alanine, L-aspartate, L-glutamate, glycine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophane, L-tyrosine and L-valine) tested did not affect growth and products of the strain. A nonprotein amino acid, γ-aminobutyrate, did not support the growth. B-vitamin requirement was not recognized.

The optimum growth conditions of strain FH042^T were tested in PY medium containing 30 mM L-arginine (PY-arginine medium) on temperatures at 5–45 °C (at 5 °C intervals), at pH 4.2, 5.3, 6.1, 6.8, 7.6, 8.2, 9.2 and 10.2 (as values verified after autoclaving), and by the addition of 0–50 g l^{-1} NaCl (at 10 g l^{-1} intervals with an exception of 5 g l^{-1}) in the medium. Bicine (Good's buffer; Dotite) (20 mM) was used to adjust the pH of the medium higher than 8.0 by using N_2 gas as the headspace. The temperature range for growth was 10–35 °C with the highest growth rate at 30 °C. The strain grew at 35 °C, but growth ceased quickly and the OD_{660} value declined. The strain grew at pH 5.3–8.2; optimum being pH 6.1–6.8. The strain grew in the medium containing NaCl up to 20 g l^{-1}; the highest growth rate was obtained without NaCl addition. The specific growth rate (μ) at the optimum conditions (30 °C and pH 6.1 without added NaCl) was 0.22–0.25 h^{-1}.

Other physiological characterization was carried out according to the methods as described by Holdeman *et al.* [12] and Ueki *et al.* [7]. Catalase, oxidase and nitrate-reducing activities were not detected. Strain FH042^T produced hydrogen sulfide. Aesculin and starch were not hydrolysed. Other physiological characteristics are presented in the species description.

Cells cultivated at 30 °C for 72 h in PY–arginine medium were used for the following chemotaxonomic analyses. Genomic DNA extracted according to the method as described by Akasaka *et al.* [15] was digested with P1 nuclease by using a YAMASA GC kit (Yamasa Shoyu, Choshi,

Japan) and its G+C content was measured by using a high-performance liquid chromatograph (Hitachi L-7400) equipped with a μBondapack C18 column (3.9×300 mm; Waters). Whole-cellular fatty acids (CFAs) were converted to methyl esters according to the method of Miller [16] and analysed by using a gas chromatograph equipped with an HP Ultra 2 column. CFAs were identified by equivalent chain length (ECL) at TechnoSuruga (Shizuoka, Japan) using the Sherlock Microbial Identification System (MOORE5; MIDI) [17]. Composition of cell-wall diagnostic diamino acid was analysed according to the method described by Akasaka *et al.* [15]. Respiratory isoprenoid quinones were extracted and analysed according to the method as described by Komagata and Suzuki [18] for the presence of menaquinones and ubiquinones. The genomic DNA G+C content of strain FH042^T was 44.7±0.2 mol%. The major components detected by the analysis of CFAs were $C_{18:1}\omega9c$ DMA (20.8 %), $C_{17:2}/C_{17:1}\omega9c$ (as summed feature 7, 12.9 %), $C_{16:0}$ DMA (11.7 %) and $C_{14:0}$ (11.5 %). Various even-numbered compounds ($C_{12:0}$–$C_{18:0}$) were detected as minor components (Table S2). The peptidoglycan of cell wall contained *meso*-diaminopimelic acid as a diagnostic diamino acid. Neither menaquinones nor ubiquinones were detected.

DNA was extracted from cells as described previously [15]. An almost full-length of 16S rRNA gene was PCR amplified using a primer set of 27f and 1546r [19]. The PCR-amplified 16S rRNA gene was sequenced by using an ABI Prism Big-Dye Terminator cycle sequencing ready reaction kit and ABI Prism 3730 automatic DNA sequencer (Applied Biosystems). Multiple alignments of the sequences with reference sequences in GenBank were performed with the BLAST program [20]. Phylogenetic trees were reconstructed by using the neighbour-joining (NJ) method [21] of the CLUSTAL W program [22] and with the maximum-likelihood (ML) program of the PHYLIP 3.695 package [23]. All gaps and unidentified base positions in the alignments were excluded before sequence assembly.

Analysis of the almost-complete 16S rRNA gene sequence (1460 bp) of strain FH042^T assigned the strain to the class *Clostridia* in the phylum *Firmicutes*. Clone GCP_T (HQ433571) from shrimp pond water in Thailand showed an almost identical sequence (99.7 % sequence similarity considering five gaps). The most closely related validly described species to strain FH042^T was a strictly anaerobic bacterium, *Anaerovorax odorimutans* DSM 5092^T [24, 25] in the family XIII *Incertae Sedis* [11] within the order *Clostridiales*, with sequence similarity of 95.1 %. The family XIII currently includes two genera *Anaerovorax* and *Mogibacterium*; *Anaerovorax odorimutans* is the only described species of the genus *Anaerovorax* and the genus *Mogibacterium* contains five species (*Mogibacterium pumilum*, *Mogibacterium diversum, Mogibacterium neglectum, Mogibacterium timidum=Eubacterium timidum* and *Mogibacterium vescum*) [11, 26]. The sequence similarities of strain FH042^T with the *Mogibacterium* species were all around 89 %. Several species within the genus *Eubacterium* [27] were related to strain FH042^T with sequence similarities of 91–92 %. The most closely related organism, but not yet validly described species, was *Clostridium* species ('*Clostridium aminobutyricum*') DSM 2634 [28] with 95.9 % sequence similarity. Based on the analysis of 16S rRNA sequences, Collins *et al.* [29] originally classified '*Clostridium aminobutyricum*' in clostridia cluster XI as a relatively distinct line. But almost all members in cluster XI have been renamed and currently assigned to the family *Peptostreptococcaceae* according to reclassification of *Clostridium* species that fall outside of cluster I [11, 30, 31]. Phylogenetic trees comprised of the species in the family XIII and the *Peptostreptococcaceae* as well as *Eubacterium* species related to strain FH042^T were reconstructed (Figs 1 and S2). Although the positions of *Anaerovorax odorimutans* and '*Clostridium aminobutyricum*' were exchanged in the two trees using the NJ and ML methods, the tree topologies evaluated by the two methods were almost the same. The phylogenetic trees showed that the species in the family *Peptostreptococcaceae* formed a distinct branch from that composed of the species in the family XIII and related species.

Some characteristics of strain FH042^T were compared with those of the species in the family XIII (*Anaerovorax odorimutans* and *Mogibacterium* species) including four *Eubacterium* species related to the family XIII and '*Clostridium aminobutyricum*' (Table 1). Cells of strain FH042^T are straight or gently curved to sigmoid rods in various sizes (Fig. S1), whereas cells of *Anaerovorax odorimutans* are typical curved or crescent-shaped rods with an approximately uniform size. Cells of strain FH042^T have polar flagella, while *Anaerovorax odorimutans* have 3–5 lateral flagella on the concave side of the cell [24]. Thus, the cell type of strain FH042^T is essentially different from that of *Anaerovorax odorimutans*. *Anaerovorax odorimutans* does not utilize carbohydrates, organic acids, alcohols and proteinogenic amino acids as growth substrates and only utilizes putrescine (a kind of polyamines), γ-aminobutyrate (a nonprotein amino acid) and 4-hydoxybutyrate. The genomic DNA G+C content of *Anaerovorax odorimutans* is 29.6 % [25], which is a similar level as those of the species in the *Peptostreptococcaceae* (26–34 %) [11]. These data indicated that it is not correct to assign strain FH042^T to the genus *Anaerovorax*. *Mogibacterium* species are anaerobic, nonspore-forming, nonmotile short rods and asaccharolytic. The genomic DNA G+C contents of the five species in the genus are 41–50 mol% [11]. Cells of the four *Eubacterium* species related to the family XIII are all anaerobic, non-spore-forming, nonmotile short rods. They are asaccharolytic and the genomic DNA G+C contents are 38–48 % [27]. All species in the two genera *Mogibacterium* and *Eubacterium* have common cellular and physiological characteristics and similar values of the genomic DNA G+C contents. The genomic DNA G+C content of strain FH042^T is also within the range observed for species of these genera. Cells of '*Clostridium aminobutyricum*' are spore-forming, motile small rods. It is saccharolytic, and produces acids (acetate and butyrate) and

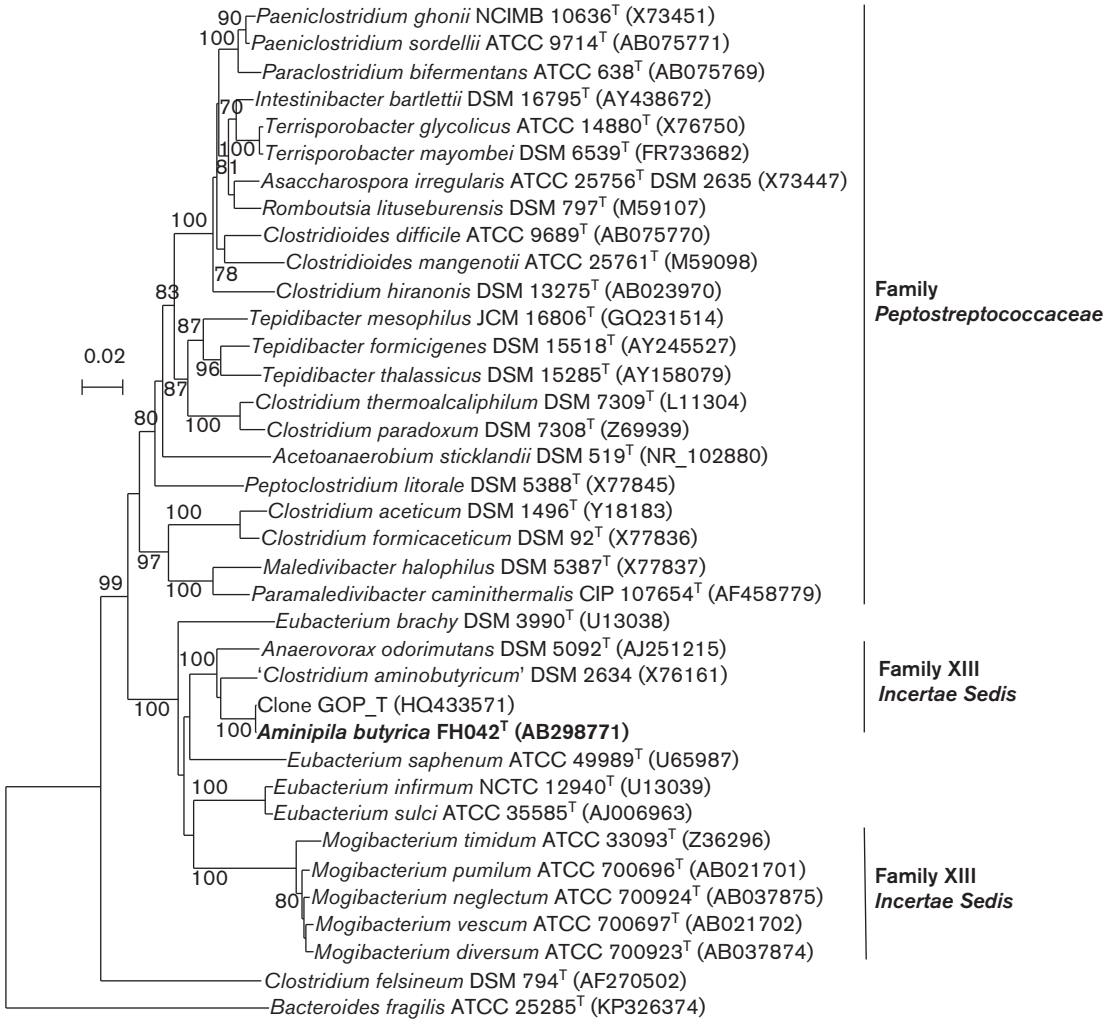

Fig. 1. Neighbour-joining tree showing the phylogenetic relationship of *Aminipila butyrica* FH042^T to related members in the family XIII and the family *Peptostreptococcaceae* in the order *Clostridiales* based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) above 70 % are shown at branch nodes. The sequence of *Bacteroides fragilis* ATCC 25285^T was used as the outgroup. Bar, 2 % estimated difference in nucleotide sequence.

gas from various carbohydrates [28]. The saccharolytic property of '*Clostridium aminobutyricum*' is exceptional in the species within the family XIII including strain FH042^T and the related *Eubacterium* species.

In addition to low 16S rRNA gene sequence similarities with all related validly described species, phenotypic characteristics of the closest species, *Anaerovorax odorimutans*, were distinctly different from those of strain FH042^T. It has been proposed that the genus *Clostridium* should be restricted to species belonging to clostridial cluster I (*Clostridium sensu stricto*) (*Clostridium butyricum* and related species) [11, 29, 32]. The 16S rRNA gene sequence similarity (95.1 %) between FH042^T and *Anaerovorax odorimutans* DSM 5092^T is almost within the threshold value for defining bacterial genera (94.5–95.0 %) recommended by Yarza *et al.* [33]. Thus, based on the data, we propose the novel genus *Aminipila* gen. nov. in the family XIII in the order *Clostridiales* to accommodate strain FH042^T as *Aminipila butyrica*. The novel species expanded the diversity of the members in the *Clostridiales* as well as insight for the proteolytic or aminolytic bacterial consortium in anaerobic digestion of organic matter.

DESCRIPTION OF *AMINIPILA* GEN. NOV.

Aminipila (A.mi.ni.pi'la. N.L. neut. n. *aminum* amine; L. fem. n. *pila* pillar; N.L. fem. n. *Aminipila* an amino acid-decomposing pillar).

Strictly anaerobic, motile rods. Gram-stain-positive. Chemoorganotroph and fermentative. Does not utilize carbohydrates. Utilizes amino acids and produces volatile fatty

Table 1. Differential characteristics of the species in the family XIII in the *Clostridiales*

Strain or species: 1, FH042^T; 2, *Anaerovorax odorimutans* NorPut1^T [24, 25]; 3, *Mogibacterium* species [11, 26]; 4, *Eubacterium* species (four species in Fig. 1) [27]; 5. *'Clostridium aminobutyricum'* [28]. +, Positive; −, negative; NR, not reported.

Characteristic	1	2	3	4	5
Cell morphology	Straight to gently curved rods	Curved or crescent-shaped rods with rounded ends	Short rods	Short rods	Small rods
Cell size (μm)	0.7–1.0×3.0–40	0.7–0.8×1.9–2.7	0.2–0.8×1.0–3.1	0.4–1.0×1.0–7.0	0.4–0.8×1.2–1.7
Gram-staining	+	− (Gram-positive type cell wall)	+	+	+
Motility	+ (1–2 polar flagella)	+ (3–5 lateral flagella on the concave side of the cell)	−	−	+
Spore	− (+*)	−	−	−	+
Utilization of growth substrate					
Carbohydrates	−	−	−	−	+
Proteinogenic amino acids	L-Arginine, L-Lysine, L-Serine	−	NR	NR†	−
γ-Aminobutyrate	−	+	NR	NR	+
Major products	Acetate, butyrate	Acetate, butyrate	Phenylacetate	Acetate, butyrate	Acetate, butyrate
Major CFAs	$C_{18:1}\omega9c$ DMA, $C_{17:2}$/$C_{17:1}\omega9c$ $C_{16:0}$ DMA, $C_{14:0}$	NR	NR	NR	NR
Genomic DNA G+C content (mol%)	44.7	29.6	41–50	38–48	NR

*Thermotolerant cells.

†One species (*E. sulci*) utilizes L-threonine.

acids. Oxidase and catalase activities are negative. Compounds even-numbered fatty acids or dimethylacetal 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 XIII *Incertae Sedis* in the order *Clostridiales*. The type species is *Aminipila butyrica*.

DESCRIPTION OF *AMINIPILA BUTYRICA* GEN. NOV., SP. NOV.

Aminipila butyrica (bu.ty′ri.ca. N.L. neut. n. *acidum butyricum* butyric acid; N.L. fem. adj. *butyrica* pertaining to production of butyric acid).

Cells are straight or slightly curved to sigmoid rods with flat ends, 0.7–1.0 μm in diameter and 3.0–8.0 μm in length. Longer rods up to 40 μm often occur. Motile with polar flagella. Spore formation is not observed, but thermotolerant cells are present. Colonies on PY4S agar are translucent with smooth surface. B-vitamins are not required for growth. Grows at 10–35 °C (optimum 30 °C), pH 5.3–8.2 (optimum pH 6.1–6.8) and 0–20 g l^{-1} of NaCl (optimum 0 g l^{-1}). Grows in PY medium and produces acetate and butyrate. Does not utilize carbohydrates and organic acids (arabinose, ribose, xylose, fructose galactose, glucose, mannose, rhamnose, sorbose, cellobiose, lactose, maltose, melibiose, sucrose, trehalose, melezitose, raffinose, carboxymethylcellulose, cellulose, dextrin, glycogen, inulin, pectin, starch, xylan, dulcitol, inositol, mannitol, sorbitol, aesculin, amygdalin, salicin, glycerol, fumarate, lactate, malate, pyruvate and succinate). Utilizes L-arginine, L-lysine and L-serine. Weakly utilizes casamino acids. Produces acetate and butyrate from L-arginine and L-lysine, and acetate from L-serine. Produces a trace amount of H_2 from L-arginine. Does not utilize other amino acids (L-alanine, L-aspartate, L-glutamate, glycine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophane, L-tyrosine, L-valine and γ-aminobutyrate). Final pH values after growth with L-arginine are 7.7 and 5.2 with L-lysine. Oxidase, catalase and nitrate-reducing activities are negative. Hydrogen sulfide is produced. Aesculin, starch and gelatin are not hydrolysed. Indole, lecithinase and lipase are not produced. Milk is unchanged and no growth occurs in chopped meat broth. The genomic DNA G+C content is 44.7±0.2 mol%. $C_{18:1}\omega9c$ DMA, $C_{17:2}$/$C_{17:1}\omega9c$ (as summed feature), $C_{16:0}$ DMA and $C_{14:0}$ are detected as major components by the cellular fatty acid analysis. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid as a diagnostic amino acid. Does not have any respiratory quinones.

The type strain, FH042^T (=JCM 31555^T=DSM 103574^T) was isolated from a methanogenic reactor treating waste from cattle farms in Japan.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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