

Caproiciproducens galactitolivorans gen. nov., sp. nov., a bacterium capable of producing caproic acid from galactitol, isolated from a wastewater treatment plant

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A strictly anaerobic, Gram-stain-positive, non-spore-forming, rod-shaped bacterial strain, designated BS-1^T, was isolated from an anaerobic digestion reactor during a study of bacteria utilizing galactitol as the carbon source. Its cells were 0.3–0.5 µm × 2–4 µm, and they grew at 35–45 °C and at pH 6.0–8.0. Strain BS-1^T produced H₂, CO₂, ethanol, acetic acid, butyric acid and caproic acid as metabolic end products of anaerobic fermentation. Phylogenetic analysis, based on the 16S rRNA gene sequence, showed that strain BS-1^T represented a novel bacterial genus within the family *Ruminococcaceae*, *Clostridium* Cluster IV. The type strains that were most closely related to strain BS-1^T were *Clostridium sporosphaeroides* KCTC 5598^T (94.5 %), *Clostridium leptum* KCTC 5155^T (94.3 %), *Ruminococcus bromii* ATCC 27255^T (92.1 %) and *Ethanoligenens harbinense* YUAN-3^T (91.9 %). Strain BS-1^T had 17.6 % and 20.9 % DNA–DNA relatedness values with *C. sporosphaeroides* DSM 1294^T and *C. leptum* DSM 753^T, respectively. The major components of the cellular fatty acids were C₁₆:₀ dimethyl aldehyde (DMA) (22.1 %), C₁₆:₀ aldehyde (14.1 %) and summed feature 11 (iso-C₁₇:₀ 3-OH and/or C₁₈:₂ DMA; 10.0 %). The genomic DNA G + C content was 50.0 mol%. Phenotypic and phylogenetic characteristics allowed strain BS-1^T to be clearly distinguished from other taxa of the genus *Clostridium* Cluster IV. On the basis of these data, the isolate is considered to represent a novel genus and novel species within *Clostridium* Cluster IV, for which the name *Caproiciproducens galactitolivorans* gen. nov., sp. nov. is proposed. The type species is BS-1^T (=JCM 30532^T and KCCM 43048^T).

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Abbreviations: DMA, dimethyl aldehyde; FAMES, fatty acid methyl esters.

The GenBank accession number for the 16S rRNA gene sequence of strain BS-1^T is FJ805840.

One figure and one table are available with the online Supplementary Material.

Municipal sewage is treated for the removal of organic compounds, phosphate and nitrogen at wastewater treatment facilities, before it is discharged into a receiving body of water. Anaerobic digestion is an important wastewater treatment process, in which organic compounds are digested by diverse anaerobic micro-organisms. However, a limited number of bacteria have been reported in the activated sludge of the anaerobic digestion reactor system (Blackall *et al.*, 1998; Hofman-Bang *et al.*, 2003;

Hugenholtz *et al.*, 1998; Kampfer *et al.*, 1996; Oda *et al.*, 1981; Snaird *et al.*, 1997; Toerien, 1967; Vainio *et al.*, 1997).

In the present study, we report on a strictly anaerobic, galactitol, algal biomass-utilizing, bacterial strain, BS-1^T, isolated from an activated sludge sample, which was collected from a municipal wastewater treatment facility in Seoul, Republic of Korea (Jeon *et al.*, 2010). The strain was isolated from galactitol-containing media, with an inoculum of sludge treated with heat (100 °C, 30 min) for the selection of heat-stable, spore-forming bacteria (Jeon *et al.*, 2010). One of the isolates, strain BS-1^T, produced caproic acid, a valuable linear C₆ carboxylic acid that can be used in the fields of bioenergy and biochemistry. The strain is considered a novel taxon of the family *Ruminococcaceae*, *Clostridium* Cluster IV, according to phylogenetic analysis performed on the basis of 16S gene sequence homology. Here, we describe polyphasic analyses to elucidate the taxonomic position of strain BS-1^T.

Anoxic culture conditions were maintained using the Hungate technique for anaerobic cultivation and was applied to all bacterial cultures in this study (Hungate, 1950). Cell culture on agar plates was performed in an anaerobic glove box (Coy) containing H₂/CO₂/N₂ (5:5:90, by vol.). Modified CAB medium (mCAB) was used as the basal medium (Kim *et al.*, 1984). mCAB comprised the following constituents (all g l⁻¹ distilled water): yeast extract, 4; tryptone, 1; KH₂PO₄, 1.5; K₂HPO₄, 1.5; L-asparagine, 0.5; MgSO₄·7H₂O, 0.1; MnSO₄·H₂O, 0.1; FeSO₄·7H₂O, 0.015; NaCl, 0.1; L-cysteine-HCl, 0.25. The pH was adjusted to 7.0 before autoclaving. Strain BS-1^T was cultured routinely on mCABGa or mCABGI, which was mCAB containing 10 g l⁻¹ galactitol or 10 g l⁻¹ glucose, respectively. A modified cooked meat medium (BD Diagnostic Systems) [supplemented with (all g l⁻¹) galactitol, 4; yeast extract, 5; glucose, 4; cellobiose, 1; soluble starch, 1 and maltose, 1] was used for the comparisons of fatty acid methyl esters (FAMES) and for the analysis of the metabolites of strain BS-1^T and closely related type strains. Cells were cultured without shaking for 3 days at 37 °C and used for the extraction of FAMES and for the analysis of metabolic end products.

Cell morphology and metabolic end products were analysed after cultivation of cells in 60 ml serum bottles containing 20 ml mCABGa and modified cooked meat broth, respectively. A seed culture (2.5 %, v/v) was inoculated in each medium and cultivated for 2 days unless otherwise specified.

Strain BS-1^T was stored as a glycerol suspension (20 %, w/v) at -70 °C. *Escherichia coli* KCTC 2441^T was received from the Korean Collection for Type Cultures (KCTC) and used as a reference strain for genomic DNA G+C content analysis. Two closely related type strains in *Clostridium* Cluster IV, *Clostridium sporosphaeroides* KCTC 5598^T (=DSM 1294^T) and *Clostridium leptum* KCTC 5155^T (=DSM 753^T), were received from KCTC for DNA-DNA hybridization assessments and comparison of physiological

characters such as FAMES, metabolic end products, and enzyme products using API test kits (bioMérieux). API 50 CH was used for evaluating the carbohydrates utilised by strain BS-1^T. API ZYM and Rapid ID 32A provided differences between the most closely related strains at the biochemical level. API tests were performed according to the manufacturer's instruction. Strain BS-1^T was deposited into the Korea Culture Center of Microorganisms (KCCM) as KCCM 43048^T and the Japan Collection of Microorganisms (JCM) as JCM 30532.

The 16S rRNA gene sequence of strain BS-1^T was compared with sequences available from GenBank using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), the Ribosomal Database Project (Maidak *et al.*, 2001) and the EzTaxon server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; Kim *et al.*, 2012), to determine the approximate phylogenetic affiliation. The 16S rRNA gene sequences of strain BS-1^T and closely related type strains were aligned using CLUSTAL X software (Thompson *et al.*, 1997). Phylogenetic trees were reconstructed with the neighbour-joining (NJ; Saitou & Nei, 1987), maximum-likelihood (ML) and maximum-parsimony (MP) methods, which were implemented in the MEGA 5 program (Tamura *et al.*, 2011). The topology of the neighbour-joining phylogenetic tree was evaluated via bootstrap analysis (Felsenstein, 1985), based on 1000 replications.

The genomic DNA G+C content of strain BS-1^T was determined using the HPLC method described by Mesbah *et al.* (1989). Genomic DNA of strain BS-1^T was extracted according to the method described by Sambrook & Russell (2001); a sample was hydrolysed with nuclease P1, and then dephosphorylated with alkaline phosphatase. The resultant mixture of nucleosides was analysed by HPLC (Young-lin SP930D) equipped with a reversed-phase column (Waters symmetry C18).

DNA-DNA hybridization was performed based on the method described by Kim *et al.* (2006) and Meinkoth & Wahl (1984). Genomic DNA was transferred to a nylon membrane (Hybond-N+; Amersham). For prehybridization and hybridization, the membrane was incubated for 1 h and 12 h, respectively, at 40 °C. Membrane-bound DNA was detected using a DIG High Prime DNA Labelling and Detection starter kit II (Roche Molecular Biochemicals). After washing, the membrane was exposed to autoradiography film (Hyperfilm-ECL; Amersham) for 10 min and signal intensities were determined using the Quantity one program (Bio-Rad). The signal intensity produced by self-hybridization was considered 100 %; the relative intensities of the genomic DNA of other strains were determined in terms of similarity to this signal intensity and were also expressed as percentages.

To obtain FAMES, saponification and methylation were conducted for cellular fatty acid and the FAMES formed were extracted by solvents. The process was performed according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 2001),

and analysis was performed using a gas chromatograph (GC; Agilent 6890n) equipped with a cross-linked methyl siloxane column (HP-1), and identified using the Microbial Identification Sherlock software package (MIDI).

Metabolic end products in aqueous culture broth were analysed using GC (Agilent 6890) equipped with a flame-ionization detector or a thermal conductivity detector, as described previously (Jeon *et al.*, 2008). For analysis of CO₂ and H₂ in the gas phase, 100 µl of gas sample from the headspace of the culture bottle was collected in a gas-tight syringe (Hamilton) and injected into a gas chromatograph equipped with a thermal conductivity detector. Argon was used as a carrier gas at a flow rate of 20 ml min⁻¹. The operational temperatures of the oven, the injector port and the detector were 50 °C, 100 °C and 200 °C, respectively. For analysis of the liquid-phase fermentation products such as ethanol and acetic acid, the culture broth was filtered using a 0.22 µm filter and injected into a GC flame-ionization detector equipped with an HP-INNOWax column (30 m × 320 µm × 0.25 µm; Agilent). Helium was used as the carrier gas at a flow rate of 1 ml min⁻¹. The oven temperature was programmed to increase from 50 °C to 170 °C at a rate of 1 °C min⁻¹. The injector and detector temperatures were both set to 250 °C.

The morphology of the cell was observed using cells cultured in mCABGa broth for 2 to 20 days at 40 °C with shaking (150 RPM). Gram staining was performed using a Gram stain set (BD Difco). The morphology of live cells was observed using a light microscope (Nikon E600; Nikon) and a scanning electron microscope (FEI XL-30). Heat and ethanol tests were used for observing spores (Burns *et al.*, 2011; Burns & Minton, 2011).

Strain BS-1^T showed enhanced growth in medium containing acetic acid (data not shown); therefore, optimum pH and temperature for growth were tested in mCABGa containing 3 g l⁻¹ acetic acid. To evaluate growth at various temperatures, strain BS-1^T was incubated at 25 °C, 30 °C, 37 °C, 40 °C, 45 °C and 50 °C. The initial pH of media varied from pH 5.0–10.0 in 0.5 unit increments; the pH was adjusted with sodium hydroxide and hydrochloric acid. The 16S rRNA gene sequences of strain BS-1^T were subjected to comparative analysis with those of closely related reference strains. On the basis of a consensus 16S rRNA gene sequence of 1356 bp, a phylogenetic tree rooted with *Bacillus subtilis* NCBI 3610 was reconstructed (Fig. 1). It showed that strain BS-1^T is affiliated to the *Clostridium* rRNA cluster

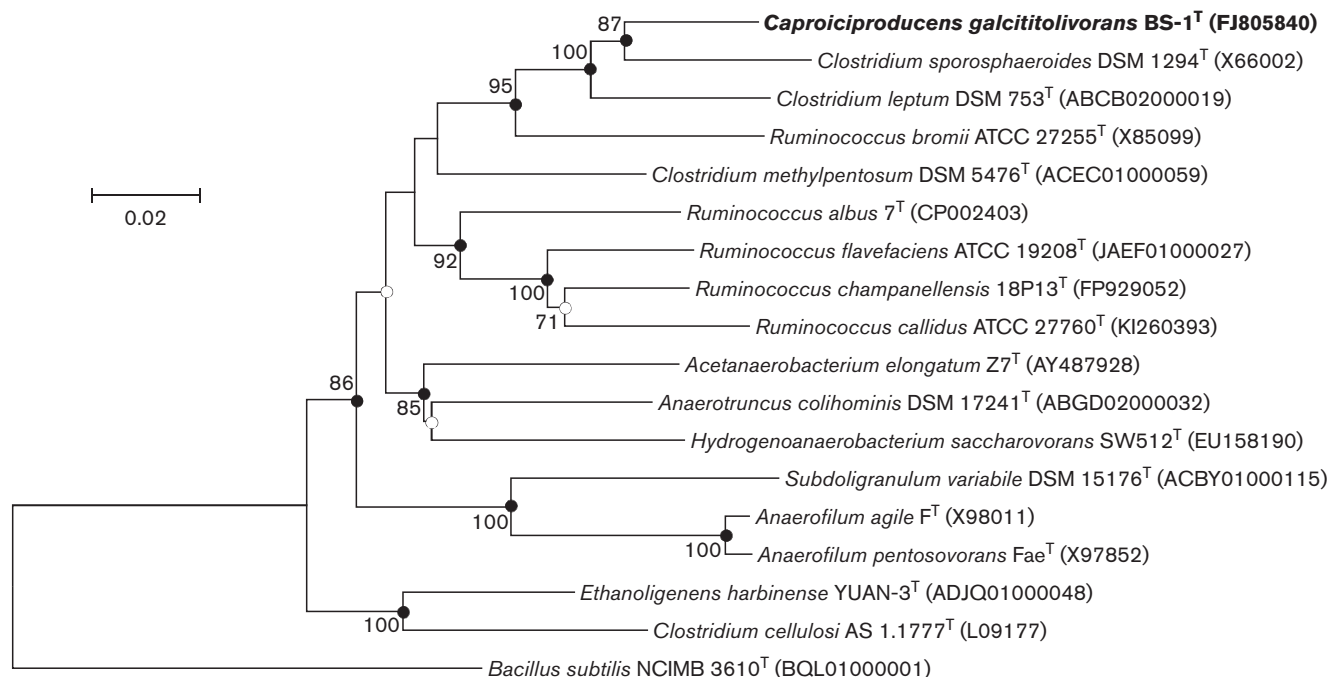


Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain BS-1^T within closely related taxa in *Clostridium* Cluster IV. GenBank accession numbers of the 16S rRNA gene sequences are given in parentheses. Black circles indicate that the corresponding branches were recovered both by the maximum-likelihood and maximum-parsimony methods. White circles indicate that the corresponding branches were recovered only in one of the above methods. Bootstrap values (based on 1000 replications) greater than or equal to 700 are shown as percentages at each node. *Bacillus subtilis* NCIMB 3610^T was used as the outgroup. Bar, 0.02 substitutions per nucleotide position.

IV, designated by Collins *et al.* (1994). *Clostridium* cluster IV may represent a suprageneric or family group in terms of sequence divergence; the members of this cluster are phenotypically heterogeneous and exhibit a broad range of chromosomal DNA G+C contents (Collins *et al.*, 1994).

In a neighbour-joining (Saitou & Nei, 1987) tree, strain BS-1^T was closely related to *C. sporosphaeroides* DSM 1294^T and *C. leptum* DSM 753^T. The topology was similar to that found in maximum parsimony and maximum-likelihood trees (data not shown). In terms of 16S rRNA gene sequence similarity, strain BS-1^T showed the highest similarity to *C. sporosphaeroides* KCTC 5598^T (=DSM 1294^T; 94.5 %), with the next highest similarity to *C. leptum* KCTC 5155^T (=DSM 753^T; 94.3 %), *Ruminococcus bromii* ATCC 27255^T (92.1 %) and *Ethanoligenens harbinense* YUAN-3^T (91.9 %). The great sequence divergence indicated that strain BS-1^T could represent a novel taxon in *Clostridium* cluster IV, in particular in the family *Ruminococcaceae* Rainey 2010 (www.bacterio.net/ruminococceae.html; Euzéby, 2010; Rainey, 2009).

The genomic DNA G+C content of strain BS-1^T was 50.0 mol%. Although strain BS-1^T is closely related to *C. sporosphaeroides* KCTC 5598^T (=DSM 1294^T) and *C. leptum* KCTC 5155^T, strain BS-1^T could be clearly differentiated from these strains, especially in terms of its DNA G+C content. Strain BS-1^T showed less than 70.0 % DNA–DNA similarity with the most closely related type species, and 17.6 % and 20.9 % DNA–DNA similarities with *C. sporosphaeroides* KCTC 5598^T and *C. leptum* KCTC 5155^T, respectively.

Strain BS-1^T produced ethanol (0.11 g l⁻¹), acetic acid (0.58 g l⁻¹), butyric acid (0.39 g l⁻¹), and caproic acid (0.69 g l⁻¹) as the end products of fermentation when cultured in modified cooked meat medium. H₂ and CO₂ were also produced during the growth of strain BS-1^T. The growth of strain BS-1^T on mCAB showed different patterns when carbon sources were added. In mCABGa, growth and caproic acid production were observed, whereas in mCABGl, only slight growth and caproic acid production were observed. In addition, the growth of strain BS-1^T and production of caproic acid were enhanced when other anaerobic volatile fatty acid-producing bacteria were co-cultured (Jeon *et al.*, 2010). In mCABGa broth, viable cells of strain BS-1^T and pH rapidly decreased compared with those grown in mCABGl broth. This growth inhibition may be related to the rapid pH drop accompanied by acetic acid production in glucose-containing medium (data not shown).

Strain BS-1^T was Gram positive, strictly anaerobic rods that measured 2.0–4.0 µm in length and 0.3–0.5 µm in width on mCABGa broth (Fig. S1, available in the online Supplementary Material). When the carbohydrate utilization of strain BS-1^T was observed using API 50CH, the strain produced

acids from glycerol, L-arabinose, L-ribose, D-xylose, L-galactose, D-glucose, D-fructose, D-mannose, dulcitol, glucosamine, D-cellobiose, starch, glycogen, D-tagatose and L-fucose.

Spores were not observed in cells cultured in mCABGa. Colonies of strain BS-1^T are circular and translucent on mCABGa and on modified cooked meat medium plates. The diameter of the colonies was <1.0 mm after 8 days at 37 °C in the anaerobic chamber. Strain BS-1^T grew at 35–45 °C and optimally at 40 °C, but did not grow at temperatures below 30 °C or above 50 °C. The initial pH range of media that allowed growth of strain BS-1^T was pH 6.0–8.0; the optimal pH was 6.5–7.5. The differentiating characteristics of strain BS-1^T and other related taxa are shown in Table 1. The other physiological and biochemical properties of strain BS-1^T are given in the species description.

The cellular fatty acid compositions and metabolic end products of strain BS-1^T and those of closely related type strains in *Clostridium* Cluster IV were determined using cells grown in a modified cooked meat medium. The growth of strain BS-1^T was very slow in any medium not containing galactitol. Therefore, comparison of the cellular fatty acid composition was performed with cultures grown in galactitol, glucose, cellobiose, soluble starch and maltose. Strain BS-1^T contained 43.7 % of dimethyl aldehyde (DMA), a specific analogue of the fatty acids present in anaerobic bacteria. The major cellular fatty acids of strain BS-1^T that were grown on modified cooked meat medium were C_{16:0} DMA (22.1 %), C_{16:0} aldehyde (14.1 %) and summed feature 11 (iso-C_{17:0} 3OH and/or C_{18:2} DMA; 10.0 %). The cellular fatty acid profile clearly differentiated strain BS-1^T from the closely related strains *C. sporosphaeroides* KCTC 5598^T and *C. leptum* KCTC 5155^T. Some distinct fatty acids such as anteiso-C_{17:0}, C_{18:1}ω9c, C_{18:1}ω9c DMA, C_{18:1}ω11c DMA, summed feature 7 (C_{17:2} and/or C_{17:1}ω8c) and summed feature 8 (C_{17:1}ω9c and/or C_{17:2}) are unique to strain BS-1^T (Table 2).

C. leptum and *C. sporosphaeroides* are spore-forming anaerobic bacteria (Chen & Dong, 2004; Yutin & Galperin, 2013). In contrast, strain BS-1^T does not form spores and contains unique cellular fatty acids such as C_{18:1} cis 9 DMA (7.6 %) and C_{18:1} cis 11 DMA (4.6 %), which are not observed in *C. leptum* and *C. sporosphaeroides*. Moreover, there was a significant difference in the G+C content between BS-1^T (50.0 %) and the closest taxon, *C. sporosphaeroides* (27.0 %). In addition, biochemical tests using API kits produced different patterns, as shown in Table S1; each of the four strains compared produced distinct metabolites during anaerobic fermentation.

The genotypic and phenotypic distinctiveness of strain BS-1^T, when it was compared to closely related type strains, suggests that strain BS-1^T represents a novel genus within the *Clostridium* Cluster IV, for which the name *Caproiciproducens galactitolivorans* sp. nov. is proposed.

Table 1. Comparison of the phenotypic characteristics of strain BS-1^T and some closely related strains

Strains: 1, strain BS-1^T; 2, *C. sporosphaeroides* (Cato *et al.*, 1986); 3, *C. leptum* (Moore *et al.*, 1976); 4, *R. bromii* (Moore *et al.*, 1972); 5, *E. harbinense* (Xing *et al.*, 2006). Metabolic end products of BS-1^T, *C. sporosphaeroides* KCTC 5598^T, and *C. leptum* KCTC 5155^T were determined in this study from cells that were cultured for 3 days at 37 °C in modified cooked meat medium. Data are from this study, Moore *et al.* (1976), Rainey (2009), Cato *et al.* (1986), Moore *et al.* (1972) and Xing *et al.* (2006). +, Positive; –, negative.

Characteristics	1	2	3	4	5
Cell shape	Rod	Rod	Rod	Coccus	Rod
Spore	–	+	+	–	–
Metabolic end products					
Propionic acid	–	+	–	–	–
Butyric acid	+	+	–	–	–
Caproic acid	+	–	–	–	–
G+C content (mol%)	50	27	51	39–40	47.8

Description of *Caproiciproducens* gen. nov.

Caproiciproducens (Ca.pro.i.ci.pro.du'cens. N.L. n. *acidum* caproic acid; L. part. adj. *producens* producing; N.L. masc. n. *Caproiciproducens* the bacteria producing caproic acid).

Strictly anaerobic bacteria. Cells are Gram-stain-positive, non-spore-forming bacilli. Carbohydrate utilization was observed with glycerol, L-arabinose, L-ribose, D-xylose, L-galactose, D-glucose, D-fructose, D-mannose, dulcitol, glucosamine, D-cellobiose, starch, glycogen, D-tagatose and L-fucose. The major metabolic end products are acetic acid, butyric acid, caproic acid and H₂. The major cellular fatty acids are C_{16:0} DMA and C_{16:0} aldehyde. Phylogenetically, the genus represents a distinct lineage in *Clostridium* Cluster IV. The type species is *Caproiciproducens galactitolivorans*.

Description of *Caproiciproducens galactitolivorans* sp. nov.

Caproiciproducens galactitolivorans (ga.lac.ti.to.li.vo'rans. N.L. n. *galactitol* -olis, galactitol; L. part. adj. *vorans* devouring; N.L. part. adj. *galactitolivorans* galactitol-devouring, utilizing galactitol).

Strictly anaerobic bacilli. Cells are Gram-stain-positive, non-spore-forming. Colonies grown on mCABGa and modified cooked meat medium plate are punctiform, circular and translucent. Single cells measure 0.3–0.5 × 2.0–4.0 μm. Growth occurs over the pH range 6.0–8.0 (optimally at pH 7.5), and at temperatures of between 35 °C and 45 °C (optimally at 45 °C). Glucose and galactitol are metabolized as carbon sources. The growth of the type strain is enhanced when other anaerobic strains producing ethanol, acetic acid, or

Table 2. Fatty acid methyl ester compositions of strain BS-1^T and two closely related strains of species of the genus *Clostridium*

Strains: 1, BS-1^T; 2, *C. sporosphaeroides* KCTC 5598^T; and 3, *C. leptum* KCTC 5155^T. All data were obtained from the present study. Cells were cultured without shaking for 3 days at 37 °C on modified cooked meat broth. Values are percentages of total fatty acids. Fatty acids comprising less than 1 % of the total in all strains are not listed. –, Not detected; DMA, dimethyl aldehyde.

Fatty acid	1	2	3
Straight-chain fatty acids			
C _{12:0}	–	4.33	–
C _{13:0}	–	1	–
C _{14:0}	3.2	4.5	2.1
C _{15:0}	–	–	1.2
C _{16:0}	3.1	1.9	7.0
C _{14:0} DMA	5.4	8.9	2.1
C _{16:0} DMA	22.1	35.9	12.7
C _{18:0} DMA	4.0	2.5	1.4
C _{16:0} aldehyde	14.1	22.6	4.0
C _{18:0} aldehyde	3.1	1.8	–
Branched fatty acids			
iso-C _{13:0}	–	0.8	1.8
iso-C _{14:0}	–	–	2.0
iso-C _{15:0}	–	–	28.8
iso-C _{15:0} DMA	–	–	14.0
anteiso-C _{15:0} DMA	–	–	1.6
anteiso-C _{15:0}	–	–	8.9
anteiso-C _{17:0}	5.9	–	–
Unsaturated fatty acids			
C _{16:1} ω9c	–	1.2	–
C _{18:1} ω9c	1.1	–	–
C _{18:1} ω9c DMA	7.6	–	–
C _{18:1} ω11c DMA	4.6	–	–
C _{18:2} ω9c, ω12c	1.1	1.4	1.0
Summed features*			
1	2.5	4.3	1
3	–	–	4.9
5	2.4	4.7	1.5
7	5.6	–	–
8	3.5	–	–
11	10.0	1.1	–

*Summed features represent groups of two or three fatty acids that could not be separated by gas chromatography using the MIDI system. Summed feature 1 contained C_{13:1}ω12c and/or C_{14:0} aldehyde. Summed feature 3 contained unknown ECL 13.570 and/or iso-C_{15:0} aldehyde. Summed feature 5 contained C_{15:0} DMA and/or C_{14:0} 3OH. Summed feature 7 contained C_{17:2} and/or C_{17:1}ω8c. Summed feature 8 contained C_{17:1}ω9c and/or C_{17:2}. Summed feature 11 contained iso-C_{17:0} 3OH and/or C_{18:2} DMA.

butyric acid are co-cultured. As fermentation end products, H₂, CO₂, ethanol, acetic acid, butyric acid and caproic acid are produced from modified cooked meat medium.

Using the API Rapid ID 32A, positive reactions are observed for α -galactosidase, α -glucosidase and glutamic acid decarboxylase. Using the API ZYM kit, positive reactions are observed for alkaline phosphatase, esterase lipase (C8), acid phosphatase, naphthol-AS-B1-phosphohydrolase, β -glucosidase and α -fucosidase. The major cellular fatty acids are C_{16:0} DMA, C_{16:0} aldehyde and summed features 11 (iso-C_{17:0} 3OH and/or C_{18:2} DMA).

The type strain, BS-1^T (=JCM 30532^T=KCCM 43048^T), was isolated from activated sludge from an anaerobic digestion reactor. The genomic DNA G+C content of the type strain is 50.0 mol%.

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