Syntrophomonas cellicola sp. nov., a spore-forming syntrophic bacterium isolated from a distilled-spirit-fermenting cellar, and assignment of *Syntrophospora bryantii* to *Syntrophomonas bryantii* comb. nov.

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A spore-forming, anaerobic, syntrophic fatty-acid-oxidizing bacterium, strain 19J-3^T, was isolated from a distilled-spirit-fermenting cellar in Hebei Province, China. The cells were slightly curved rods with a spore at the end of the cell. The optimal temperature for growth was around 37 °C and growth occurred in the range 25–45 °C. The pH range for growth was 6.5–8.5 and the optimum pH was 7.0–7.5. Crotonate was the only substrate that allowed the strain to grow in pure culture. However, the strain could oxidize saturated fatty acids with four to nine carbon atoms syntrophically in co-culture with *Methanobacterium formicicum* DSM 1535^T. The strain was not able to utilize sulfate, sulfite, thiosulfate, DMSO, nitrate, fumarate or Fe(III) as electron acceptor. The DNA base composition was 48.8 mol% G + C. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain 19J-3^T was related to members of the family *Syntrophomonadaceae* and most closely to *Syntrophospora bryantii* DSM 3014^T (94.3 % similarity) and *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T (93.6 % similarity). Considering the phylogenetic relationship and phenotypic features, strain 19J-3^T (=CGMCC 1.5041^T = JCM 13582^T) is designated as the type strain of a novel species of the genus *Syntrophomonas*, *Syntrophomonas cellicola* sp. nov. Based on the close phylogenetic relationship between the genera *Syntrophospora* and *Syntrophomonas*, the presence of sporulation-specific genes in the genome of *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T and the description of a spore-forming member of *Syntrophomonas*, '*Syntrophomonas erecta* subsp. *sporosyntropha*', we propose the assignment of *Syntrophospora bryantii* to the genus *Syntrophomonas* as *Syntrophomonas bryantii* comb. nov.

Butyrate is an important intermediate in the anaerobic degradation of organic matter (Mackie & Bryant, 1981) and has to be degraded by consortia of at least two trophic groups (syntrophic acetogenic bacteria and hydrogen scavengers) because of the unfavourable energetics of the reactions (Schink, 1997). *Syntrophomonas wolfei* subsp. *wolfei* (McInerney *et al.*, 1979, 1981; Beaty & McInerney, 1987) was the first described syntrophic bacterium that degraded butyrate in co-culture with methanogens or hydrogen-utilizing sulfate-reducing bacteria. Since then, *Syntrophospora bryantii* (Stieb & Schink, 1985; Zhao *et al.*, 1990), *Syntrophomonas sapovorans* (Roy *et al.*, 1986), *Syntrophomonas wolfei* subsp. *saponavida* (Lorowitz *et al.*, 1989), *Thermosyntropho lipolytica* (Svetlitsnyi *et al.*, 1996), *Syntrophus aciditrophicus* (Jackson *et al.*, 1999), *Smithella*

propionica (Liu *et al.*, 1999), *Syntrophothermus lipocalidus* (Sekiguchi *et al.*, 2000), *Syntrophomonas curvata* (Zhang *et al.*, 2004) and *Syntrophomonas erecta* (Zhang *et al.*, 2005) have been isolated and characterized. Recently, '*Syntrophomonas erecta* subsp. *sporosyntropha*', which produced spores in co-culture with methanogens, was described (Wu *et al.*, 2006). In this paper, we describe the isolation and characterization of a sporulated *Syntrophomonas* strain with Gram-positive cell-wall structure, strain 19J-3^T, which was obtained from a distilled-spirit-fermenting cellar in Hebei Province, China.

Methanobacterium formicicum DSM 1535^T and *Desulfovibrio* strain G11 were kindly provided by Dr Alfons Stams (Department of Microbiology, Wageningen University, The Netherlands).

A pre-reduced basal medium described by McInerney *et al.* (1979) was used for isolation and routine cultivation. The

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Syntrophomonas cellicola* 19J-3^T is DQ288691.

gas phase was N_2 gas (1.01×10^5 Pa) except that *Methanobacterium formicicum* DSM 1535^T was cultivated with H_2/CO_2 (80:20, 1.25×10^5 Pa). All inoculations and transfers were done with syringes and needles. Incubations were at 37 °C and in dark unless indicated. The purity of co-cultures and pure cultures was examined periodically by monitoring the cell morphology under a bright-field microscope and the absence of growth in peptone-yeast extract-glucose (PYG) medium (Britz & Tracey, 1983).

The procedures used to examine the morphology, nutrition and growth properties, to extract genomic DNA and to determine the G + C content of the novel strain were those described by Zhang *et al.* (2005).

The 16S rRNA gene was amplified by PCR and sequenced using the method described by Weisburg *et al.* (1991). 16S rRNA gene sequences of strain 19J-3^T and reference strains in GenBank were aligned using CLUSTAL X program (version 1.83). Phylogenetic trees were constructed using UPGMA, minimum evolution and maximum-parsimony methods implemented in the MEGA3 program and the topology of the phylogenetic trees was evaluated by bootstrap analysis of 1000 datasets.

A sample from the walls of a distilled-spirit-fermenting cellar was inoculated into pre-reduced medium with 20 mM butyrate to enrich for anaerobic butyrate-degrading consortia. After repeated roll-tube isolations (Hungate, 1969) in butyrate agar medium which contained a 5 % (v/v) culture of *Methanobacterium formicicum* DSM 1535^T and 5 % (v/v) of the enrichment culture, a biculture producing methane from butyrate but not in PYG medium was obtained. This biculture formed brownish colonies of 0.6–1.0 mm in diameter in roll tubes and showed green fluorescence under 420 nm light. Only two kinds of cell morphologies were observed in the biculture, one was a straight *Methanobacterium formicicum*-like rod and the other was a slightly curved, spore-forming rod. To purify the spore-forming strain, the culture was pasteurized twice at 90 °C for 25 min and, after pasteurization, 0.5 ml heated culture and 0.5 ml H_2/CO_2 -grown *Methanobacterium formicicum* DSM 1535^T were inoculated into butyrate-containing basal medium and incubated at 37 °C until methane was detected. The monoculture of the butyrate-degrading syntrophic strain was purified by inoculating the biculture into medium with 20 mM crotonate as sole substrate instead of butyrate and 10 mM 2-bromoethanesulfonic acid to inhibit growth of the methanogen, and the isolate was designated as strain 19J-3^T. When growing on crotonate agar medium in pure culture, tiny white colonies, 0.2–0.3 mm in diameter, were formed.

Cells of strain 19J-3^T were slightly curved rods with rounded ends, $0.4\text{--}0.5 \times 3.0\text{--}10.0$ µm, occurring singly or in clumps. An oval spore was formed at the end of the cell (Fig. 1a) and poly-β-hydroxyalkanoate was accumulated (Fig. 1b), which was confirmed by cytochemical stain (Burdon, 1946).

Fig. 1. Photomicrographs of cells of strain 19J-3^T grown in pure culture on crotonate. (a) Phase-contrast light micrograph. Arrows point to sporulated cells. Bar, 6 µm. (b) Electron micrograph of an ultrathin section showing the typical Gram-positive wall structure and the presence of poly-β-hydroxyalkanoate (PHA); the arrow indicates a PHA granule. Bar, 0.3 µm.

The Gram reaction was negative to weakly positive; however, a Gram-positive cell wall ultrastructure was observed in ultrathin sections of strain 19J-3^T (Fig. 1b). A single flagellum was inserted in the midpoint of the cell (not shown).

Strain 19J-3^T degraded 20 mM butyrate into about 40 mM acetate and 4.87 mM methane within 14 days in co-culture with *Methanobacterium formicicum* DSM 1535^T and degraded 20 mM butyrate within 10.5 days in co-culture with *Desulfovibrio* strain G11. The strain neither grew nor produced methane on butyrate alone, whereas it degraded butyrate in an artificially constructed co-culture with *Methanobacterium formicicum* DSM 1535^T. Straight-chain fatty acids with four to nine carbons were degraded by co-cultures of strain 19J-3^T with *Methanobacterium formicicum*

DSM 1535^T or *Desulfovibrio* strain G11; however, neither branched-chain fatty acids such as isobutyrate and isovalerate nor benzoate were degraded. Among the substrates used, fatty acids with even numbers of carbons were converted into acetate and H₂, while those with odd numbers of carbons were converted into propionate, acetate and H₂, implying that β -oxidation of fatty acids occurred. None of the following substrates were used by strain 19J-3^T as potential electron acceptors for butyrate degradation: sodium sulfate (20 mM), sodium thiosulfate (20 mM), sulfur (20 mM), DMSO (20 mM), sodium nitrate (20 mM) and sodium fumarate (20 mM).

Crotonate was the only compound tested that supported growth of strain 19J-3^T in pure culture, and 24.5 mM crotonate was degraded to about 10.4 mM butyrate and 23.3 mM acetate in 4.5 days, with electron and carbon recoveries of $90.0 \pm 1.3\%$ and $89.4 \pm 1.6\%$, respectively. The doubling time of strain 19J-3^T was 6.5 h when grown on 20 mM crotonate at 37 °C. The molar growth yield of strain 19J-3^T with crotonate was 5.0–5.6 g dry weight mol⁻¹. Growth and acid formation were not observed on the following substrates: yeast extract (0.5%), tryptone (1%), glucose (20 mM), ribose (20 mM), xylose (20 mM), pyruvate (20 mM) and fumarate (20 mM).

The temperature range for growth of both the co-culture on butyrate and pure culture on crotonate was 25–45 °C, with optimal growth at 37–40 °C, and the pH for growth of both ranged from 6.5 to 8.5 with an optimal initial pH of 7.0–7.5. The NaCl concentration range tolerated was 0–450 mM (better growth below 100 mM). No growth was observed in air.

The G + C content of the genomic DNA from strain 19J-3^T was determined as 48.8 mol%.

A phylogenetic tree including strain 19J-3^T and other members of the family *Syntrophomonadaceae* was constructed (Fig. 2) based on a consensus length of 1421 bp of the 16S rRNA genes. Phylogenetically, strain 19J-3^T is most closely related to *Syntrophospora bryantii* DSM 3014^T

(94.3% similarity) and *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T (93.6% similarity), with 91.8–94.0% similarity to other *Syntrophomonas* species.

By a combination of phylogenetic relationships and phenotypic characteristics, strain 19J-3^T could represent a novel species of the genus *Syntrophomonas*. Morphologically, strain 19J-3^T formed spores in both co-culture and monoculture, while most other *Syntrophomonas* species did not form spores in the same medium and growth conditions. Comparison of substrate utilization patterns with those of other *Syntrophomonas* strains showed that strain 19J-3^T differed from *Syntrophomonas wolfei* subsp. *saponavida* DSM 4212^T, *Syntrophomonas sapovorans* DSM 3441^T and *Syntrophomonas curvata* DSM 15682^T by using only fatty acids with four to nine carbon atoms, from *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T by not using isoheptanoate and from *Syntrophomonas erecta* DSM 16215^T and '*Syntrophomonas erecta* subsp. *sporosyntrophae*' by using pelargonate. Strain 19J-3^T differed from *Syntrophospora bryantii* by a difference of 11.2 mol% in G + C content and by not utilizing caprate, undecanoate or 2-methylbutyrate. Table 1 shows the characteristics of strain 19J-3^T that distinguish it from other taxa. Therefore, we propose strain 19J-3^T as the type strain of a novel species of the genus *Syntrophomonas*, *Syntrophomonas cellicola* sp. nov.

Clostridium bryantii was transferred to the new genus *Syntrophospora* by Zhao *et al.* (1990) on the basis of sporulation and the presence of a Gram-positive cell-wall ultrastructure, which differentiated it from *Syntrophomonas wolfei* subsp. *wolfei*, a non-spore-former with a Gram-negative cell wall ultrastructure. Although *Syntrophomonas* species possess a complex cell-wall ultrastructure indicative of a Gram-negative cell wall, the lack of lipopolysaccharides confirms their Gram-positive cell-wall structure. The recently completed genome sequence of *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T (<http://genome.ornl.gov/microbial/swol/>) showed the presence of sporulation-specific genes in the genome, such as *spo0A*, a master regulator in bacterial sporulation found exclusively in spore-forming bacteria (Brown *et al.*, 1994). We found that all

Fig. 2. Phylogenetic tree of strain 19J-3^T and related organisms based on a 1421 bp fragment of the 16S rRNA gene. The tree was rooted with the sequence from *Clostridium butyricum* ATCC 43755^T and was constructed by using the UPGMA method. Solid circles indicate that the corresponding nodes (groups) are also recovered in minimum evolution and maximum-parsimony methods. Numbers at nodes represent percentage levels of bootstrap support based on the UPGMA method of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 2% sequence divergence.

Table 1. Characteristics of strain 19J-3^T and other mesophilic syntrophic bacteria in the family *Syntrophomonadaceae*

Strains: 1, *Syntrophomonas cellicola* sp. nov. 19J-3^T; 2, *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T (unless indicated, data from McInerney *et al.*, 1979, 1981; Beaty & McInerney, 1987); 3, *Syntrophospora bryantii* DSM 3014^T (Stieb & Schink, 1985; Zhao *et al.*, 1990); 4, *Syntrophomonas sapovorans* DSM 3441^T (Roy *et al.*, 1986); 5, *Syntrophomonas curvata* DSM 15682^T (Zhang *et al.*, 2004); 6, *Syntrophomonas erecta* DSM 16215^T (Zhang *et al.*, 2005); 7, '*Syntrophomonas erecta* subsp. *sporosyntropha*' JCM 13344 (Wu *et al.*, 2006); 8, *Syntrophomonas wolfei* subsp. *saponavida* DSM 4212^T (Lorowitz *et al.*, 1989). ND, No data available. All strains are positive for metabolism of butyrate to caprylate in co-culture with methanogens.

Characteristic	1	2	3	4	5	6	7	8
Cell width (µm)	0.4–0.5	0.5–1.0	0.4	0.5	0.5–0.7	0.6–0.9	0.5–0.7	0.4–0.6
Cell length (µm)	3.0–10.0	2.0–7.0	3.0–6.0	2.5	2.3–4.0	2.0–8.0	4–14	2.0–4.0
Gram type	+	–	+	–	–	–	–	–
Flagella	1	2–8	0	2–?	1–3	2–5	0	2–4
DNA G + C content (mol%)	48.8	45.1*	37.6	ND	46.6	43.2	40.6	ND
Optimum pH	7.0–7.5	ND	6.6–7.5	7.3	7.5	7.8	7.0	ND
Optimum temperature (°C)	37–40	35–37	28–34	25–45	35–37	37–40	37–40	ND
Spore formation								
Co-culture	+	–	+	–	–	–	+	–
Monoculture	+	–	+	–	–	–	–	–
Substrate utilization in co-culture with methanogens								
2-Methylbutyrate	–	–	+	–	–	–	–	–
Pelargonate	+	ND	+	+	+	–	–	+
Caprate	–	ND	+	+	+	–	–	+
Undecanoate	–	ND	+	+	+	–	–	+
Laurate	–	–	–	+	+	–	–	+
Myristate	–	–	–	+	+	–	–	+
Isoheptanoate	–	+	–	–	–	–	–	–

*Data from Zhang *et al.* (2004).

recognized species of the genus *Syntrophomonas* contain a homologue of the *spo0A* gene of *Clostridium* and *Bacillus* species (C. Wu, X. Liu and X. Dong, unpublished data). Furthermore, sporulating *Syntrophomonas* strains were reported recently (Wu *et al.*, 2006). Therefore, *Syntrophomonas* species all have the potential to form spores, which is in agreement with their phylogenetic placement within the low-G + C-content Gram-positive bacteria. The inability to detect sporulation with previously isolated *Syntrophomonas* strains is probably due to the lack of suitable culture conditions. There is growing recognition that sporulation per se is not necessarily a good indicator of relatedness (Cook *et al.*, 1991; Farrow *et al.*, 1995). In addition, Collins *et al.* (1994) proposed to merge the genera *Syntrophospora* and *Syntrophomonas*, as the level of evolutionary divergence between *Syntrophospora bryantii* and *Syntrophomonas wolfei* subsp. *wolfei* was only about 6%. Therefore, we propose to assign *Syntrophospora bryantii* to the genus *Syntrophomonas* as *Syntrophomonas bryantii* comb. nov.

Emended description of the genus *Syntrophomonas* McInerney *et al.* 1982

The genus description is the same as that given by Lorowitz *et al.* (1989) except that some strains form

spores in co-culture with methanogens on fatty acids or in pure culture on crotonate. Isolated from anaerobic environments such as aquatic sediments, digester sludge, rumen digest, rice field mud and shallow marine sediments.

Description of *Syntrophomonas cellicola* sp. nov.

Syntrophomonas cellicola (cel.li.co'la. L. n. *cella* a storeroom for wine and food; L. suff. *-cola* from L. n. *incola* an inhabitant, dweller; N.L. n. *cellicola* an inhabitant of a storeroom, indicating that the type strain was originally isolated from a distilled-spirit-fermenting cellar).

Cells are Gram-variable, slightly curved rods, 0.4–0.5 × 4–7 µm, spore-forming and with one laterally inserted flagellum. The type strain grows in pure culture only on crotonate. In syntrophic association with *Methanobacterium formicicum* DSM 1535^T or *Desulfovibrio* strain G11, the organism uses saturated fatty acids with four to nine carbon atoms by β-oxidation. Cells grow between 25 and 45 °C (optimum 37 °C) and at pH 6.5–8.5 (optimum pH 7.0–7.5). The genomic DNA G + C content is 48.8 mol%.

The type strain is 19J-3^T (=CGMCC 1.5041^T=JCM 13582^T), isolated from a distilled-spirit-fermenting cellar in Hebei Province, China.

Description of *Syntrophomonas bryantii* comb. nov.

Basonym: *Clostridium bryantii* Stieb and Schink 1985.

The description of *Syntrophomonas bryantii* is that given for *Syntrophospora bryantii* by Zhao *et al.* (1990). The type strain is strain CuCal^T [=DSM 3014A^T (co-culture with *Desulfovibrio* sp. E70) =DSM 3014B^T (co-culture with *Methanospirillum hungatei* M1h)].

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