Syntrophobacter sulfatireducens sp. nov., a novel syntrophic, propionate-oxidizing bacterium isolated from UASB reactors

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Two obligate anaerobes, TB8106^T and WZH410, which degraded propionate in syntrophic association with methanogens, were isolated from two upflow anaerobic sludge blanket reactors, one treating brewery wastewater and the other bean curd wastewater. The strains were Gram-negative, non-spore-forming and non-motile. Cells were egg-shaped, with a size of 1.0–1.3 × 1.8–2.2 µm. Growth was observed at 20–48 °C and pH 6.2–8.8. Both strains converted propionate to acetate and methane in co-culture with methanogens, and grew on propionate plus sulfate in pure culture, with a doubling time of 52–55 h at 37 °C. Sulfate and thiosulfate both served as electron acceptors for propionate degradation. The DNA G + C contents of the two strains were 58.5 and 58.7 mol%, respectively. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the strains were closely related to a propionate-oxidizing syntrophic bacterium, *Syntrophobacter fumaroxidans* DSM 10017^T (94.7 % similarity). However, the novel strains could not ferment fumarate, and grew at a more alkaline pH range than *Syntrophobacter fumaroxidans*. Moreover, the novel isolates had obviously higher growth rates on propionate plus sulfate (0.12 day⁻¹) than *Syntrophobacter fumaroxidans* DSM 10017^T (0.024 day⁻¹). Therefore, a novel species, *Syntrophobacter sulfatireducens* sp. nov., is proposed, with strain TB8106^T (=AS 1.5016^T = DSM 16706^T) as the type strain.

In methanogenic environments, complex organic matter is degraded completely to CO₂ and CH₄ by the association of several trophic micro-organisms (Zehnder, 1978). Propionate, one of the central intermediates in this process, is further degraded by the combined action of acetogenic bacteria and methanogenic archaea, due to the unfavourable energetics under standard thermodynamics. The methanogens make propionate oxidation energetically feasible by keeping the concentration of the products H₂ and/or formate extremely low (Stams, 1994; Schink, 1997). So far, seven syntrophic propionate-oxidizing bacterial species have been described. The four mesophilic species form a cluster within the Gram-negative δ -subclass of the *Proteobacteria*, closely related to the mesophilic sulfate reducers.

They are *Syntrophobacter wolinii* (Boone & Bryant, 1980; Wallrabenstein *et al.*, 1994), *Syntrophobacter fumaroxidans* (Harmsen *et al.*, 1998), *Syntrophobacter pfennigii* (Wallrabenstein *et al.*, 1995) and *Smithella propionica* (Liu *et al.*, 1999). Members of the genus *Syntrophobacter* can use sulfate as an electron acceptor and oxidize propionate via the methylmalonyl CoA pathway (Harmsen *et al.*, 1993; Houwen *et al.*, 1990; Plugge *et al.*, 1993), whereas *Smithella propionica*, which grows axenically on crotonate, uses a six-carbon-intermediate pathway to oxidize propionate (Liu *et al.*, 1999; de Bok *et al.*, 2001). Unlike the mesophilic species, the three described thermophilic propionate-oxidizing species are all Gram-positive, spore-forming bacteria (Nilsen *et al.*, 1996; Imachi *et al.*, 2002; Plugge *et al.*, 2001). In this study, we describe two novel mesophilic syntrophic propionate-oxidizing strains, TB8106^T and WZH410. Phylogenetic analysis indicates that both strains are members of the genus *Syntrophobacter*. However, they are not closely related to strains of recognized *Syntrophobacter* species.

Strains TB8106^T and WZH410 were isolated from the granular sludge of two mesophilic upflow anaerobic sludge blanket (UASB) reactors, one treating brewery wastewater

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Abbreviation: UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Syntrophobacter sulfatireducens* TB8106^T is AY651787.

A transmission electron micrograph of a cell of *Syntrophobacter sulfatireducens* TB8106^T is available as supplementary material in IJSEM Online.

in Tsinghua University and the other treating bean curd wastewater in Wangzhihe bean curd manufactory (Beijing, China). *Methanospirillum hungatei* DSM 864^T and *Syntrophobacter fumaroxidans* DSM 10017^T were kindly provided by Dr Alfons Stams, Department of Microbiology, Wageningen University, The Netherlands.

A pre-reduced basal medium was used as described by Stams *et al.* (1992), and yeast extract (0.02 %, w/v) and tryptone (0.02 %, w/v) were added to stimulate growth. Routine cultivation was in anaerobic tubes (18 × 180 mm), sealed with butyl rubber stoppers. A gaseous atmosphere of N₂/CO₂ (80:20, 1.01 × 10⁵ Pa) was used, except that H₂/CO₂ (80:20, 1.25 × 10⁵ Pa) was used for cultivating methanogens. All inoculations and transfers were done with syringes and needles, and all cultures were incubated at 37 °C in the dark. For the isolation of co-cultures, direct serial dilution and the Hungate roll-tube technique (Hungate, 1969) were used, with 20 mM propionate as the sole carbon source. For the isolation of pure cultures of propionate-degrading bacteria, 20 mM propionate plus 20 mM sulfate were used. The purity of the strains in co-culture and pure culture was checked by microscopic examination, and by colony formation on solid media, as well as by the absence of growth in rich medium such as tryptone-peptone-yeast extract-glucose liquid medium (Holdeman *et al.*, 1977).

Cell morphology was examined under a light microscope (Olympus BH-2) and an electron microscope (Hitachi H-600A). For electron microscopy studies, cells were negatively stained with uranyl acetate.

In order to determine the optimum growth conditions for strains TB8106^T and WZH410, the basal medium with 20 mM propionate plus 20 mM sulfate and a 10 % inoculum were used. Growth at various pH values was determined in media adjusted with 1 M HCl or NaOH. Temperature profiles were determined by using a water bath (Guangming Medical Instrument Plant, Beijing) at 15 to 55 °C, at 1 °C intervals (pH 7.5). To determine the NaCl tolerance, 0–1000 mM NaCl was added to the medium. The generation time of the strains was determined by monitoring the OD₆₀₀ of the culture at 37 °C at 1-day intervals for up to 60 days. Growth under the various conditions given above was determined by monitoring the OD₆₀₀ after cultivation for up to 60 days.

To determine the substrate range of the strains in pure culture and in co-culture with methanogens, sterilized substrates were added to the basal medium to a final concentration of 20 mM (unless indicated otherwise), instead of propionate. To determine the electron acceptors for propionate degradation of the methanogen-free culture, each of the tested compounds (20 mM) was added to the propionate medium. After inoculation, the cultures were incubated for up to 90 days. Utilization of substrates by the strains was determined by monitoring the OD₆₀₀ of the culture, substrate depletion and product formation. Fatty

acids and CH₄ were detected by using a gas chromatograph (GC-14B; Shimadzu), as described previously (Chen & Dong, 2004). All tests were performed in duplicate with a 10 % inoculum.

Genomic DNA was extracted and purified from strains TB8106^T and WZH410 grown on propionate plus sulfate, as described by Marmur (1961). The mol% G + C content of DNA was determined by the thermal denaturation method (Marmur & Doty, 1962), by using a DU800 spectrophotometer (Beckman) with *Escherichia coli* K-12 as the reference. The 16S rRNA gene was amplified and sequenced according to Chen & Dong (2004). Sequencing was performed by Sangon Biological Engineering Technology Service, Shanghai, China, using ABI PRISM Big Dye Terminator cycle sequencing ready reaction kits (Perkin Elmer) and an ABI PRISM 377XL DNA sequencer. The 16S rRNA gene sequence of strain TB8106^T was submitted to GenBank and EMBL to search for similar sequences using the BLAST algorithm. The best matching sequences were retrieved from the database and aligned, and similarity analysis was performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Phylogenetic trees were constructed by using the neighbour-joining, maximum-likelihood and maximum-parsimony methods, implemented in the MEGA 2 program (Kumar *et al.*, 2001) and the PHYLIP package (Felsenstein, 1993). The topologies of the resultant tree were evaluated by bootstrap analysis (Felsenstein, 1985), based on 1000 resamplings.

DNA–DNA hybridization was performed at 73 °C on the basis of DNA–DNA liquid reassociation rate (De Ley *et al.*, 1970), using a DU800 spectrophotometer (Beckman).

Propionate-degrading methanogenic enrichments were developed from anaerobic digester sludge by diluting the sludge at a ratio of 1 : 10 with the basal medium plus 20 mM sodium propionate. After 3 weeks of incubation at 37 °C, the enriched cultures degraded propionate and produced large amounts of CH₄. The enrichments were subcultured six times, and then the roll-tube technique was applied with propionate-containing agar medium. After incubation for 10 weeks, colonies were visible in the roll tubes. Large colonies at dilutions of 10⁻⁴ and 10⁻⁵ that emitted green fluorescence at a wavelength of 420 nm were picked and inoculated into the propionate medium. This roll-tube procedure was repeated several times over a period of 2 years, until two co-cultures that produced methane from propionate were obtained. The co-cultures consisted of two kinds of cell with different morphologies: one was egg-shaped and the other resembled *Methanobacterium formicicum* cells. Colonies of the co-cultures were yellowish, circular and 1.5–2.0 mm in diameter, and emitted green fluorescence at 420 nm after 10 weeks of incubation. The propionate oxidizers were further purified by inoculating the co-culture onto medium containing 20 mM propionate plus 20 mM sulfate; 10 mM 2-bromoethane sulfonate was also added to inhibit the methanogen. After being subcultured 12 times on this medium, monocultures

designated as strains TB8106^T and WZH410 were obtained. Hardly any growth of the strains as a pure culture was observed on solid medium, and propionate could not be degraded in the absence of sulfate, whereas propionate was degraded to acetate and CH₄ in an artificially constructed co-culture with *Methanospirillum hungatei* DSM 864^T after 50–60 days.

Cells of strains TB8106^T and WZH410 were non-motile, Gram-negative, egg-shaped, and 1.0–1.3 × 1.8–2.2 µm in size (see Supplementary Figure in IJSEM Online). Spore formation was never observed. The strains were strictly anaerobic; no growth occurred when exposed to air. The temperature range for growth was 20–48 °C, with optimum growth occurring at 37 °C. The pH range for growth was 6.2–8.8, with optimum growth occurring at pH 7.0–7.6. NaCl was tolerated up to a concentration of 100 mM (best growth occurred at concentrations below 50 mM). Addition of 0.02 % yeast extract and tryptone stimulated, but was not necessary for, growth. In pure culture on propionate plus sulfate, 20 mM propionate was oxidized to 20 mM acetate within 12–15 days. The doubling time of strain TB8106^T was 52–55 h at 37 °C, and the growth rate was approximately 0.12 day⁻¹. The growth yield of strain TB8106^T on propionate plus sulfate was 3.3 g dry weight (mol propionate)⁻¹.

Sulfate and thiosulfate were utilized as electron acceptors for propionate oxidation, but not sulfur, nitrate or fumarate. Strain TB8106^T utilized sulfite, whereas WZH410 did not. Both strains fermented pyruvate in pure culture. The following substrates were not used in either pure or co-culture: H₂/CO₂ (80:20, v/v), formate, H₂ plus fumarate, formate plus fumarate, acetate, butyrate, crotonate, succinate, fumarate, oxaloacetate, malate, citrate, malonic acid, glutarate (10 mM), alanine (10 mM), glycine (10 mM),

asparagine (10 mM), benzoate (10 mM), methanol, ethanol, 1-propanol, 1-butanol, glucose (10 mM), fructose (10 mM), xylose (10 mM) and arabinose (10 mM). Propionate was the sole substrate for the co-culture.

The G + C contents of the genomic DNA of strains TB8106^T and WZH410 were 58.5 and 58.7 mol%, respectively. The partial 16S rRNA gene sequence (500 bp) similarity between strains TB8106^T and WZH410 was 99.94 %, indicating that they belonged to the same species.

To ascertain the phylogenetic position of strain TB8106^T, the complete 16S rRNA gene sequence (1553 bp) was compared with the most similar sequences retrieved from GenBank. On the basis of a consensus 1361 bp of the 16S rRNA gene sequence, a phylogenetic tree (Fig. 1), rooted with *E. coli* ATCC 11775^T, was constructed. The tree clearly showed that strain TB8106^T clustered within the genus *Syntrophobacter*, in the δ-subclass of the *Proteobacteria*, and was most closely related to strain HP1.1 (Zellner *et al.*, 1996) (99.2 % similarity), a highly purified propionate-degrading culture, and *Syntrophobacter fumaroxidans* DSM 10017^T (94.7 % similarity) and *Syntrophobacter pfennigii* DSM 10092^T (94.6 % similarity).

Strains TB8106^T and WZH410 resembled the syntrophic, propionate-oxidizing species *Syntrophobacter fumaroxidans*, *Syntrophobacter pfennigii* and *Syntrophobacter wolinii* in that growth occurred on propionate in co-culture with methanogens and sulfate reduction was observed in pure culture. However, distinct differences in phenotypic characteristics were observed (Table 1). In monoculture, *Syntrophobacter wolinii* and *Syntrophobacter fumaroxidans* both fermented fumarate, and *Syntrophobacter fumaroxidans* also utilized fumarate as an electron acceptor for propionate oxidation. *Syntrophobacter pfennigii* utilized lactate not only in

Fig. 1. Phylogenetic dendrogram of *Syntrophobacter sulfatireducens* TB8106^T and related species based on 16S rRNA gene sequence similarity. The tree was rooted with *E. coli* ATCC 11775^T and was constructed using the neighbour-joining method. Solid circles indicate that the corresponding nodes (groups) were also recovered using maximum-likelihood and maximum-parsimony methods. Numbers at nodes represent percentage levels of bootstrap support, based on a neighbour-joining analysis of 1000 resampled data sets. GenBank accession numbers of 16S rRNA sequences are given in parentheses. Bar, 2 % sequence divergence.

Table 1. Characteristics that can be used to differentiate *Syntrophobacter sulfatireducens* TB8106^T from other species of the genus *Syntrophobacter*

Species/strains: 1, *Syntrophobacter sulfatireducens* TB8106^T; 2, *Syntrophobacter* strain HP1.1 (data from Zellner *et al.*, 1996); 3, *Syntrophobacter fumaroxidans* DSM 10017^T (Harmsen *et al.*, 1998); 4, *Syntrophobacter pfennigii* DSM 10092^T (Wallrabenstein *et al.*, 1995); 5, *Syntrophobacter wolinii* DSM 2805^T (Boone & Bryant, 1980; Wallrabenstein *et al.*, 1994; Harmsen *et al.*, 1998). –, Negative; +, positive; ND, not determined.

Characteristic	1	2	3	4	5
Cell width (µm)	1.0–1.3	1.25–1.5	1.1–1.6	1.0–1.2	0.6–1.0
Cell length (µm)	1.8–2.2	1.75–2.5	1.8–2.5	2.2–3.0	1.0–4.5
Motility	–	–	–	+	–
Gas vacuoles	–	–	–	+	–
DNA G+C content (mol%)	58.5	56.7	60.6	57.3	ND
Growth pH:					
Range	6.2–8.8	ND	6.0–8.0	6.2–8.0	ND
Optimum	7.0–7.6	ND	7.0	7.0–7.3	ND
Growth temperature (°C):					
Range	20–48	20–42	20–40	20–37	ND
Optimum	37	40	37	37	ND
Utilization in pure culture of:					
Lactate	–	–	–	+	ND
Fumarate	–	ND	+	–	+
Pyruvate	+	ND	+	–	+
Utilization in co-culture with methanogens of:					
Lactate	–	ND	–	+	ND
Propanol	–	ND	–	+	ND
Growth rate on propionate plus sulfate (day ^{–1})	0.12	ND	0.024	0.07	0.062

monoculture, but also in methanogenic co-culture. All these features were not shared by strains TB8106^T and WZH410. Furthermore, strains TB8106^T and WZH410 had obviously higher growth rates on propionate plus sulfate (0.12 day^{–1}) than *Syntrophobacter fumaroxidans* DSM 10017^T (0.024 day^{–1}). In addition, the novel isolates grew well at 48 °C and pH 8.8, a higher growth temperature and pH than those for other members of the genus *Syntrophobacter*. DNA–DNA hybridization between strain TB8106^T and *Syntrophobacter fumaroxidans* DSM 10017^T showed a low genome DNA–DNA relatedness (12.5%), indicating that they belong to different species.

On the basis of the morphological and physiological differences, 16S rRNA gene sequence divergency and lower DNA–DNA relatedness between the strains described in this report and other members of the genus *Syntrophobacter*, we propose that strains TB8106^T and WZH410 should be classified as representing a novel species, for which the name *Syntrophobacter sulfatireducens* sp. nov. is proposed.

The description of the genus *Syntrophobacter* of Boone & Bryant (1980) indicated that sulfate did not serve as an electron acceptor in the energy metabolism. However, the later-described syntrophic propionate-degrading species

Syntrophobacter fumaroxidans, *Syntrophobacter pfennigii* and the novel isolates in this work were all demonstrated to be capable of coupling propionate oxidation to sulfate reduction. In addition, *Syntrophobacter wolinii* was also shown to be able to use sulfate as an electron acceptor (Wallrabenstein *et al.*, 1994). Hence, it is necessary to emend the description of the genus to emphasize the sulfate reduction capability, in accordance with its close phylogenetic relationship with sulfate reducers.

Emended description of the genus *Syntrophobacter*

The description is based on the phenotypic data of Boone & Bryant (1980), Wallrabenstein *et al.* (1994, 1995) and Harmsen *et al.* (1998).

Gram-negative, non-motile, rod- to egg-shaped cells. Endospores are not formed. Strictly anaerobic chemo-organotrophs. Mesophilic. Growth by fermentation or by reduction of sulfate to sulfide. Pure cultures oxidize propionate with sulfate as the electron acceptor. Propionate is fermented syntrophically to acetate and CO₂ in the presence of hydrogen- and/or formate-utilizing methanogens (e.g. *Methanospirillum hungatei*). The G+C content of the genomic DNA is 56.7–60.6 mol%.

The type species is *Syntrophobacter wolinii*.

Description of *Syntrophobacter sulfatireducens* sp. nov.

Syntrophobacter sulfatireducens (sul'fa'ti.re.du'cens. N.L. n. *sulfas* -atis, sulfate; L. v. *reducere* to lead back, bring back; N.L. part. adj. *sulfatireducens* reducing sulfate).

Morphology and general characteristics are as described for the genus. Egg-shaped cells, $1.0\text{--}1.3 \times 1.8\text{--}2.2$ μm in size, single, in pairs or in chains. Pure cultures oxidize propionate and pyruvate to acetate, with sulfate as electron acceptor; pyruvate is also oxidized by fermentation. Does not grow on H_2/CO_2 , formate, H_2 plus fumarate, formate plus fumarate, acetate, butyrate, crotonate, succinate, fumarate, oxaloacetate, malate, citrate, malonic acid, glutarate, alanine, glycine, asparagine, benzoate, methanol, ethanol, 1-propanol or 1-butanol. Sulfate and thiosulfate are both used as electron acceptors, but sulfur, nitrate and fumarate are not reduced. Growth is optimal at pH 7.0–7.6 and at 37 °C. The G+C content of genomic DNA is 58.5–58.7 mol%. Habitat is granular sludge of UASB reactors.

The type strain is TB8106^T (=AS 1.5016^T=DSM 16706^T), which was isolated from a UASB reactor treating brewery wastewater, in Beijing, China.

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