Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*

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A strain of anaerobic, syntrophic, propionate-oxidizing bacteria, strain LYP^T (= OCM 661^T; T = type strain), was isolated and proposed as representative of a new genus and new species, *Smithella propionica* gen. nov., sp. nov. The strain was enriched from an anaerobic digester and isolated. Initial isolation was as a monoxenic propionate-degrading co-culture containing *Methanospirillum hungatei* JF-1^T as an H₂- and formate-using partner. Later, an axenic culture was obtained by using crotonate as the catabolic substrate. The previously described propionate-degrading syntrophs of the genus *Syntrophobacter* also grow in co-culture with methanogens such as *Methanospirillum hungatei*, forming acetate, CO₂ and methane from propionate. However, *Smithella propionica* differs by producing less methane and more acetate; in addition, it forms small amounts of butyrate. *Smithella propionica* and *Syntrophobacter wolinii* grew within similar ranges of pH, temperature and salinity, but they differed significantly in substrate ranges and catabolic products. Unlike *Syntrophobacter wolinii*, *Smithella propionica* grew axenically on crotonate, although very slowly. Co-cultures of *Smithella propionica* grew on propionate, and grew slowly on crotonate or butyrate. *Syntrophobacter wolinii* and *Syntrophobacter pfennigii* grow on propionate plus sulfate, whereas *Smithella propionica* did not. Comparisons of 16S rDNA genes indicated that *Smithella propionica* is most closely related to *Syntrophus*, and is more distantly related to *Syntrophobacter*.

Keywords: *Smithella propionica*, propionate-oxidizing bacteria, anaerobic digester

INTRODUCTION

Propionate is an important intermediate in methanogenic fermentation: it is the precursor of a large fraction of methane from anaerobic digestors (Boone, 1982, 1984; Kaspar & Wuhrmann, 1978a), and its accumulation inhibits anaerobic digestion (Kaspar & Wuhrmann, 1978b). Propionate is oxidized to acetate and CO₂ (Boone, 1984; Stadtman & Barker, 1951), with the electrons generated from this oxidation ultimately transferred to methanogens that form methane by CO₂ reduction. H₂ (Boone & Bryant, 1980; Wolin, 1982) and formate (Boone *et al.*, 1989; Stams & Dong, 1995; Thiele *et al.*, 1988) are the interspecies

electron carriers for this transfer. Propionate oxidation with H₂ and formate production as the electron sink is exergonic only when H₂ and formate concentrations are very low (Boone & Bryant, 1980; McInerney & Bryant, 1981; Schink, 1997) (Table 1, equation A).

The propionate-degrading bacterium *Syntrophobacter wolinii* was first isolated in co-cultures in which one or both of these catabolic products were continuously removed by methanogens or sulfate-reducing bacteria (Boone & Bryant, 1980). More recently, *Syntrophobacter wolinii* was shown to be phylogenetically related to sulfate-reducing bacteria (Harmsen *et al.*, 1993). This led to the demonstration that *Syntrophobacter wolinii* can grow slowly on propionate with sulfate as electron acceptor, and allowed the first isolation of this bacterium in axenic culture (Wallrabenstein *et al.*, 1994). Stams *et al.* (1993) showed that propionate

The GenBank accession number for the 16S rRNA sequence of strain LYP^T is AF126282.

Table 1. Possible catabolic reactions during the anaerobic degradation of propionate

Reaction	Equation	ΔG^0 (kJ/reaction) [†]	H ₂ at equilibrium*	
			Pa	nM
I. Hydrogen-producing reactions				
A	CH ₃ CH ₂ COO ⁻ + 2H ₂ O → CH ₃ COO ⁻ + CO ₂ + 3H ₂	71.7	12.1	89.2
B	CH ₃ CH ₂ CH ₂ COO ⁻ + 2H ₂ O → 2CH ₃ COO ⁻ + H ⁺ + 2H ₂	48.3	275	2024
II. Hydrogen-consuming reactions				
C	CO ₂ + 4H ₂ → CH ₄ + 2H ₂ O	-130.7	0.317	2.33
D	CH ₃ CH ₂ COO ⁻ + CO ₂ + 3H ₂ → CH ₃ CH ₂ CH ₂ COO ⁻ + 2H ₂ O	-71.5	12.3	90.6
E(D + B)	CH ₃ CH ₂ COO ⁻ + CO ₂ + H ₂ → 2CH ₃ COO ⁻ + H ⁺	-23.3	0.025	0.18
III. Combined hydrogen-producing and hydrogen-consuming reactions				
F(B + 1/2C)	CH ₃ CH ₂ CH ₂ COO ⁻ + 1/2CO ₂ + H ₂ O → 2CH ₃ COO ⁻ + H ⁺ + 1/2CH ₄	-17.1		
G(4A + 3C)	4CH ₃ CH ₂ COO ⁻ + 2H ₂ O → 4CH ₃ COO ⁻ + CO ₂ + 3CH ₄	-105.6		
H(A + D)	2CH ₃ CH ₂ COO ⁻ → CH ₃ COO ⁻ + CH ₃ CH ₂ CH ₂ COO ⁻	0.12		
I(3F + A)	4CH ₃ CH ₂ COO ⁻ + 2CO ₂ + 2H ₂ O → 7CH ₃ COO ⁻ + 3H ⁺	1.91		

* H₂ activity for $\Delta G = 0$ with the following activities: CH₄, 0.5 atm; CO₂, 0.5 atm; acetate, propionate and butyrate at 1 mM; pH = 7.

[†] H₂, CO₂, and CH₄ in the gaseous state; H⁺ at 10⁻⁷ mol activity per kg; all other substances in aqueous solution at 1 mol of activity per kg; 25 °C (Thauer *et al.*, 1977).

enrichment cultures could also grow in the absence of syntrophic interactions by fermenting fumarate to succinate and CO₂, and demonstrated the enzymes of a fermentative pathway that includes acetyl-CoA oxidation to CO₂ (Plugge *et al.*, 1993). They were unable to grow *Syntrophobacter wolinii* axenically on fumarate because fumarate was metabolized by the sulfate-reducing bacterium present in that syntrophic culture (Stams *et al.*, 1993). In this paper, we report the isolation of the type strain of *Syntrophobacter wolinii* from its co-culture with *Desulfovibrio* strain G11 in monoxenic co-culture with *Methanospirillum hungatei* and in axenic culture with fumarate as a catabolic substrate.

Propionate degradation by *Syntrophobacter wolinii* (Houwen *et al.*, 1991), anaerobic sediments (Schink, 1985), anaerobic digestors (Robbins, 1987, 1988; Tholozan *et al.*, 1988) and other methanogenic environments (Boone, 1984; Houwen *et al.*, 1987, 1990; Koch *et al.*, 1983) occurs mainly by the methyl-malonyl pathway, with succinate as a symmetrical intermediate. This pathway leads to the production of 1 mol acetate, 1 mol CO₂ and three pairs of electrons (i.e. a total of 3 mol H₂ and formate) per mol propionate degraded (Table 1, equation A). However, some propionate in anaerobic digestors is reductively carboxylated to butyrate (Samain *et al.*, 1984; Tholozan *et al.*, 1988), and this reduction can occur in the absence of methanogenesis (Samain *et al.*, 1984). This six-electron reduction (e.g. Table 1, equation D) probably does not use H₂ as electron donor if it is a catabolic reaction, because the reaction is not exergonic at the low H₂ concentrations found in anaerobic digestors. However, the electrons to accomplish this reduction could be

generated internally, such as by propionate oxidation (Table 1, equation H) or, in part, by butyrate oxidation (Table 1, equation D).

In this paper, we report the isolation of strain LYP^T, a bacterium that syntrophically degrades propionate with butyrate as a small but significant product, and that also slowly degrades butyrate. We propose it as the type strain of *Smithella propionica* gen. nov., sp. nov. This strain's 16S rDNA sequence, whose determination is reported herein, has been used to develop primers that detect phylogenetically similar organisms in anaerobic digestors (Sauer *et al.*, 1997). Portions of this material were reported previously (Liu *et al.*, 1998).

METHODS

Source of inoculum and cultures. The source from which strain LYP^T was enriched was an up-flow anaerobic filter inoculated with digested domestic sewage sludge and operated with propionate as the major feedstock for 6 months. Cultures were obtained from the Oregon Collection of Methanogens (OCM): *Syntrophobacter wolinii* DB^T in co-culture with *Desulfovibrio* G11 (= OCM 467), *Syntrophomonas wolfeii* LYB (= OCM 65), which is a butyrate-oxidizing syntroph (Boone *et al.*, 1989), *Methanosarcina barkeri* NIH-1 (= OCM 266) was purified from *Methanosarcina barkeri* NIH (= OCM 265) (Blaylock & Stadtman, 1966) by roll-tube isolation in MS medium with 50 mM methanol. *Methanospirillum hungatei* JF-1^T (= OCM 16^T) is a H₂- and formate-utilizing methanogen.

Media and culture technique. We used the anaerobic techniques of Hungate (1969) with some modifications (Miller & Wolin, 1974; Sowers & Noll, 1995). Except where noted, we used MS medium (Boone *et al.*, 1989), an

anaerobic bicarbonate-buffered medium with 2 g l⁻¹ each of Trypticase peptone and yeast extract; MES (0.5 g l⁻¹) and Na₂S.9H₂O (0.25 g l⁻¹) were the reducing agents. MS enrichment medium was the same as MS medium except that yeast extract and Trypticase peptone were reduced to 0.5 g of each per litre. We also used MS low-nutrient medium in which the amount of yeast extract was reduced to 0.01 g l⁻¹ and Trypticase peptone was omitted. Agar media contained 18 g purified agar l⁻¹. For isolations, we placed cultures into anaerobic jars with an anoxic gas to eliminate oxygen diffusion through the stoppers, thus allowing longer anoxic incubations.

Analytical techniques. Methane was quantified by GC with flame-ionization detection (Maestrojuán & Boone, 1991) and fatty acids by GC of free acids. Samples were acidified by adding 0.1 ml 3 M phosphoric acid to 0.9 ml of sample. Injection volume was 1 µl. Acids were separated in a column (2 m long, with 2 mm i.d.) containing 10% (w/w) SP-1000 coated on Chromosorb 101. The column temperature was 105 °C and carrier-gas (N₂) flow rate was 40 ml min⁻¹. Detection was by flame ionization. Concentrations were estimated by comparison of integrated peaks to those of standards.

Analysis of growth and purity. Growth of methanogenic co-cultures was checked by methane measurement (Maestrojuán & Boone, 1991), and growth of non-methanogenic cultures was monitored by optical density at 670 nm (Spectronic 21; Milton Roy). Purity was checked routinely by inoculating fluid thioglycollate medium (Difco) and MS medium. Immediately following the initial isolation of axenic cultures, media with H₂ plus sulfate or H₂ plus CO₂ were also inoculated to check for the presence of sulfate-reducers or methanogens, as indicated. After 3–8 weeks incubation, these contamination-check cultures were visually checked for turbidity and microscopically checked for the presence of bacteria (Boone & Bryant, 1980).

Electron microscopy. Cells were centrifuged from the growth medium and resuspended in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) at room temperature and fixed for 30 min. After two buffer washes, cells were fixed in 1% osmium tetroxide in the same buffer for 1 h at 4 °C, washed in distilled water, dehydrated in an ethanol series, and embedded in low-viscosity epoxy resin for sectioning. Sections on Formvar-coated grids were poststained with uranyl acetate and lead citrate, and viewed and photographed with a Zeiss EM-10CA transmission electron microscope.

Cytochemical stain for poly-hydroxyalkanoate. Cells were stained for detection of poly-β-hydroxyalkanoate (Doetsch, 1981). A heat-fixed film of cells was stained with 0.3% Sudan black B in ethylene glycol for 15 min, rinsed in toluene, counter-stained briefly with 0.5% aqueous safranin, and dried. Stained cells were examined without a coverslip with an oil-immersion objective lens on a Nikon Optiphot II microscope using differential interference contrast optics.

Phylogenetic analysis. DNA from strain LYP^T was isolated by the chloroform/isoamyl alcohol procedure (Johnson, 1981). Approximately 20 ng DNA was used as a template for PCR amplification (Sambrook *et al.*, 1989). The PCR amplification primers (Weisburg *et al.*, 1991) were fD1 (5' AGAGTTTGATCCTGGCTCAG) and rP2 (5' ACGGCT-ACCTTGTTACGACTT). The PCR amplification products were sequenced with an Applied Biosystems model 373A DNA sequencer by using the Taq DyeDeoxy terminator

cycle sequencing method according to the manufacturer's procedures (McBride *et al.*, 1989). The following primers were used for sequencing: C (5' ACGGGCGGTGTGTAC) (Lane *et al.*, 1985), corresponding to positions 1406–1392 in the 16S rDNA nucleotide sequence of *Escherichia coli* (Brosius *et al.*, 1978); C-complement (5' GTACACACCG-CCCGT; *E. coli* positions 1392–1406); H (5' ACACGA-GCTGACGACAGCCA; positions 1075–1056); G (5' CC-AGGGTATCTAATCCTGTT; positions 800–781); G-complement (5' AACAGGATTAGATACCCTGG; positions 781–800); A (5' GTATTACCGCGG[C/G]TGCTG; positions 536–519); P (5' CTGCTTCCTCCCGTAGGAG; positions 357–339); P-complement (5' CTACGGGAGGC-AGCAG; positions 342–357); and F₂C (5' AGAGTT-TGATC[A/C]TGGCTC; positions 8–25). The assembled 16S rDNA sequences were hand-aligned with the equivalent 16S rDNA or rRNA sequences of all closely related strains found in the GenBank database. The initial sets of prealigned eubacterial sequences were obtained from the Ribosomal Database Project (Larsen *et al.*, 1993). Each set of aligned sequences was analysed for maximum-parsimony with the program PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1993) to construct the most parsimonious phylogenetic tree based on *E. coli* positions 14–676. Only the phylogenetically informative sites were considered, and alignment gaps were retained in the analysis. A heuristic search was carried out first (with standard program defaults), after which a bootstrap analysis placed confidence limits on the branch points of the resulting phylogenetic trees. Consensus phylogenetic trees for each alignment set were produced by bootstrapping at the >50% confidence limit, with 100 replications (Felsenstein, 1993). The phylogenetic position of strain LYP^T, inferred from its 16S rDNA sequence, was compared to the positions of the most closely related organisms, with successive comparisons based on the analytical results of the previous alignment.

The aligned sequences were then analysed with parsimony and distance matrix methods. The parsimony analysis was performed with the program PAUP as described above. The distance matrix analysis was carried out by using the PHYLIP package of the microcomputer programs (Felsenstein, 1993). Distances were calculated by the method of Jukes & Cantor (1969), after which phylogenetic distances were estimated with the FITCH option, which makes use of the Fitch–Margoliash criterion (Fitch & Margoliash, 1967) and some related least-squares criteria.

RESULTS

Isolation of strain LYP^T in monoxenic co-culture

MS enrichment medium with 20 mM propionate was inoculated (5%) with an anaerobic filter and incubated at 37 °C. A stable enrichment culture was developed from this culture by transferring 10% to fresh MS low-nutrient medium with 20 mM propionate. The culture was maintained by routine transfer after methane production ceased. After six such transfers, a culture in mid-exponential growth phase was serially diluted and inoculated into MS enrichment medium with 20 mM propionate. Each culture was co-inoculated with *Methanospirillum hungatei*. These dilutions were also inoculated into solidified medium in roll tubes with lawns of *Methanospirillum hungatei*, and the liquid cultures and roll-tube cultures were incubated at 37 °C.

Colonies in roll-tube media did not appear to contain propionate-degrading bacteria. However, after 8 weeks incubation, the liquid culture inoculated with 0.1 nl of original culture had grown. It contained epifluorescent curved rods appearing to be *Methanospirillum hungatei* and shorter, thicker, non-fluorescent rods. The methane formed by the culture was much less than that formed by co-cultures of *Syntrophobacter wolinii*. We used dilutions of this culture to inoculate MS roll-tube media that contained 20 mM propionate and a lawn of both *Methanospirillum hungatei* and *Methanosarcina barkeri*. After 6 months incubation, a colony was picked and inoculated into MS medium with 20 mM propionate. This culture contained short, non-epifluorescent rods that were slightly narrower than those of *Syntrophobacter wolinii*, as well as epifluorescent cells appearing to be *Methanospirillum hungatei* and *Methanosarcina barkeri*. No growth was detected when this co-culture was inoculated into MS medium without propionate or into fluid thioglycollate medium, indicating the absence of contaminants able to grow in these media. The co-culture was deposited in the Oregon Collection of Methanogens as OCM 441.

Axenic isolation of strain LYP^T with crotonate

The co-culture grew very slowly in liquid MS medium with 20 mM butyrate (5 months to complete growth) or 20 mM crotonate (6 months to complete growth). Subsequent transfers (10% of culture volume) grew faster (4–8 weeks). A crotonate-grown culture after several transfers contained fewer methanogens. It was serially diluted and inoculated into MS medium with 20 mM crotonate. The highest dilutions that grew were those that had been inoculated with 0.1 nl of the original culture. These were examined microscopically and found to contain only non-epifluorescent cells appearing to be the propionate-degrading bacterium. This culture was checked for contamination by inoculation of thioglycollate medium, MS medium and MS medium with H₂ added (to detect methanogens). The culture was axenic. It was named strain LYP^T and deposited in OCM as OCM 661^T. This culture grew in MS medium with crotonate, but it was unable to grow when yeast extract and peptones were omitted. Thus, the strain required one or more growth factors present in yeast extract or peptones.

Isolation of *Desulfovibrio*-free cultures of *Syntrophobacter wolinii*

The methods used to obtain methanogenic co-cultures of strain LYP^T were successfully applied to *Syntrophobacter wolinii*. We grew cultures of the triculture of strain LYP^T, *Desulfovibrio* strain G11 and *Methanospirillum hungatei* (Boone & Bryant, 1980) in MS mineral medium plus 20 mM propionate. After six transfers, *Methanospirillum hungatei* and *Syntrophobacter wolinii* were dominant, with very few cells of *Desulfovibrio* strain G11 evident. We inoculated dilu-

tions of this culture into MS medium with propionate as substrate, also adding 0.1 ml of a culture of *Methanospirillum hungatei* to each tube. After 2 months incubation, cultures that had been inoculated with 1 nl of original culture had grown. Cells of *Desulfovibrio* strain G11 appeared to be absent: they could not be found by microscopic examination of the culture, nor could they be found after incubation of the culture in MS medium with sulfate and H₂. The culture appeared to contain only two morphological types, those of *Methanospirillum hungatei* and of *Syntrophobacter wolinii*. This co-culture was inoculated into roll-tube medium with propionate and lawns of both *Methanospirillum hungatei* and *Methanosarcina barkeri*. After 6 months incubation, light yellow colonies formed that comprised three morphological types, attributed to *Methanospirillum hungatei*, *Methanosarcina barkeri* and *Syntrophobacter wolinii*. No growth occurred when the culture was inoculated into fluid thioglycollate medium.

Axenic isolation of *Syntrophobacter wolinii* on fumarate

A methanogenic co-culture of *Syntrophobacter wolinii* DB^T and *Methanospirillum hungatei* was obtained by serial dilution of the triculture that also contained *Methanosarcina barkeri*. This co-culture was grown for several transfers in MS medium with 20 mM fumarate. Microscopic examination revealed reduced numbers of *Methanospirillum hungatei*, so the culture was serially diluted into MS medium with 20 mM fumarate. After 8 weeks incubation, the highest dilution that grew had produced no methane. This culture contained a single morphological type appearing to be *Syntrophobacter wolinii*. Microscopic examination and contamination checks in thioglycollate medium, MS medium without additional substrates and MS medium with H₂ indicated that this was an axenic culture of *Syntrophobacter wolinii* DB^T. This culture was deposited in OCM as OCM 587^T. It grew on malate, fumarate or pyruvate, but not on propionate. However, when co-inoculated with *Methanospirillum hungatei* into MS medium with 20 mM propionate, the culture grew and formed methane in expected amounts.

Morphology

Strain LYP^T cells were elongated, slightly sinuous rods 0.5 µm in diameter and 4–5 µm in length (Fig. 1a and c). Cells as long as 10 µm were observed. Cells usually contained one to several electron-light globules. Staining with Sudan black indicated that these bodies were poly-β-hydroxyalkanoate (Fig. 1c). The cells stained Gram-negative, and the walls had a typical Gram-negative ultrastructure (Fig. 1b).

For comparison we examined *Syntrophobacter wolinii* and found important differences. Although the cells are approximately the same dimensions, and were also Gram-negative, the ends of *Syntrophobacter wolinii*

Fig. 1. Micrographs of propionate-degrading bacteria: (a) Transmission electron micrograph of longitudinal section of *Smithella propionica* (arrowheads indicate granules of poly- β -hydroxyalkanoate within the cytoplasm; bar, 1 μ m). (b) High magnification transmission electron micrograph thin-section of *Smithella propionica*, illustrating typical Gram-negative wall structure (M, plasma membrane; P, peptidoglycan; W, wall; bar, 0.1 μ m). (c) Light microscopy of Sudan black B cytochemical stain to demonstrate poly- β -hydroxyalkanoate granules in cells of *Smithella propionica* (arrowheads indicate dark granules that are positively stained; bar, 5 μ m). (d) High-magnification transmission electron micrograph thin-section of *Syntrophobacter wolinii* DBT⁺, illustrating typical Gram-negative wall structure and invaginations of plasma membrane (one at arrowhead) forming disk-like plates in cytoplasm (bar, 0.1 μ m). (e) Transmission electron micrograph of a longitudinal section of *Syntrophobacter wolinii* DBT⁺; cells tapered to flat ends; arrowheads indicate several of the cytoplasmic disks as shown in part (d) (bar, 1 μ m).

cells tapered to a blunt point (Fig. 1e). The cytoplasm of fumarate-grown cells contained numerous membrane-delimited inclusions (Fig. 1d and 1e), but these inclusions were absent in cells grown on pyruvate. The inclusions were invaginations of the plasma membrane (arrowhead, Fig. 1d) and in unusually thick sections appeared as disks when viewed face on. Gas vesicles as reported for *Syntrophobacter pfennigii* (Wallrabenstein *et al.*, 1995b) were not found.

Reconstitution of strain LYP^T co-cultures from axenic cultures

Like *Syntrophomonas wolfeii*, strain LYP^T was able to grow axenically in MS medium with 20 mM crotonate, but not with 20 mM butyrate as catabolic substrate. However, when an axenic culture of strain LYP^T was co-inoculated with *Methanogenium* strain PM or *Methanospirillum hungatei*, the co-cultures grew slowly on butyrate, and produced methane in expected quantities. Likewise, an axenic culture of strain LYP^T did not grow in MS medium with 20 mM propionate, but it grew in this medium when co-inoculated with

Methanospirillum hungatei and *Methanosarcina barkeri*. This triculture produced the stoichiometrically expected amount of methane from propionate (viz. 1.75 mol methane per mol propionate).

Propionate degradation by co-cultures of strain LYP^T

When a reconstituted co-culture of strain LYP^T and *Methanospirillum hungatei* was grown in 20 ml MS medium with 0.4 mmol propionate (20 mM), only 0.06 mmol methane was formed by 60 d incubation (Fig. 2a). This was much less than the expected amount of methane (0.3 mmol) based on the degradation stoichiometry of *Syntrophobacter wolinii* (Table 1, equation G). In this time, the culture degraded 0.3 mmol propionate, and produced 0.4 mmol acetate and 0.04 mmol butyrate; about 0.1 mmol propionate remained (Fig. 2a). This stoichiometry differed from that of *Syntrophobacter wolinii* (Boone & Bryant, 1980), but was similar to that expected if 2 mol propionate were fermented to 1 mol each of butyrate and acetate (Table 1, equation H), with most of the butyrate further degraded syntrophically (Table 1, equation F).

Fig. 2. Effect of acetate degraders and butyrate degraders on the degradation of 20 mM propionate by the propionate-degrading strain LYP^T and *Methanospirillum hungatei*. (a) Growth of strain LYP^T plus *Methanospirillum hungatei*. (b) Growth of strain LYP^T, *Methanospirillum hungatei* and *Methanosarcina barkeri*. (c) Thermodynamics of several propionate-degrading reactions at concentrations occurring during the batch growth shown in (a) (Reactions G, H and F refer to the reactions shown in Table 1). (d) Thermodynamics of several propionate-degrading reactions at concentrations occurring during the batch growth shown in (b) (Reactions G, H and F refer to the reactions shown in Table 1).

Inhibition by H₂ or formate

To determine whether inefficient H₂ (or formate) removal limited propionate oxidation by strain LYP^T, we increased the numbers of methanogens in the co-culture. We did this by pre-growing *Methanospirillum hungatei* in 20 ml medium with H₂ added; we flushed out the gas space with N₂ and CO₂ (3:7) after growth was complete. We then added 0.4 mmol propionate (20 mM) to this culture and inoculated it with the propionate-degrading co-culture. The increased numbers of methanogens had little effect on the fermentation (data not shown).

To confirm that strain LYP^T required H₂ and formate removal for propionate degradation, we added bromoethanesulfonate to inhibit the uptake of H₂ and formate by the methanogen. When the co-culture was incubated with 0.5 mM bromoethanesulfonate, methane was not produced, propionate concentration did not decrease, and no growth was detected. This finding is consistent with the inability of the *Syntrophobacter wolinii* to co-culture with *Desulfovibrio* strain G11 in

the absence of sulfate (Boone & Bryant, 1980), and with our finding that strain LYP^T did not grow axenically on propionate. However, it contrasts with experiments on sewage sludge, in which propionate conversion to butyrate continued in the presence of bromoethanesulfonate (Samain *et al.*, 1984). Growth of strain LYP^T on propionate depended on the removal of H₂ and formate by *Methanospirillum hungatei*.

Inhibition by acetate and other organic acids

The incomplete degradation of propionate by the co-culture (Fig. 2a) may have been due to inhibition by accumulating acetate. When the aceticlastic methanogen *Methanosarcina barkeri* was co-inoculated with the co-culture of strain LYP^T and *Methanospirillum hungatei*, more propionate was degraded than when *Methanosarcina barkeri* was absent (Fig. 2b). Acetate accumulated and then disappeared; ultimately, much more methane was formed than in the absence of *Methanosarcina barkeri*. Thus, the high (> 20 mM) concentrations of acetate that had accumu-

Fig. 3. Effect of temperature on growth rates of *Smithella propionica* LYP^T (□) and *Syntrophobacter wolinii* DB^T (○).

lated in co-cultures of strain LYP^T and *Methanospirillum hungatei* appeared to inhibit the extent of propionate degradation. Elevated acetate concentration also appeared to stimulate net butyrate formation, as more butyrate was formed when acetate was allowed to accumulate than when it was removed by *Methanosarcina barkeri* (Fig. 2).

Added acetate inhibited methanogenesis from propionate by strain LYP^T and *Methanospirillum hungatei*. When such a co-culture was inoculated into medium with 20 mM propionate and 10 mM acetate, the acetate inhibited the rate (by 35%) and extent (by 18%) of methanogenesis from propionate compared to cultures without added acetate. When 20 mM acetate was added, inhibition of the rate (by 66%) and extent (by 49%) of methanogenesis was more severe, and inhibition by 40 mM added acetate was nearly complete.

To determine whether inhibition was a general feature of organic acids rather than product inhibition, we tested methanogenesis from propionate in the presence of various concentrations of added organic acid salts. Methanogenesis by the triculture of strain LYP^T, *Methanospirillum hungatei* and *Methanosarcina barkeri* was uninhibited by propionate concentrations as high as 60 mM, and good growth occurred even with 120 mM propionate. Butyrate was not inhibitory at the highest concentrations tested (20 mM), but rather it slightly stimulated methanogenesis. On the other hand, 10 mM isobutyrate strongly inhibited methanogenesis from propionate.

Other catabolic substrates

Strain LYP^T grew axenically on 10 mM crotonate, producing approximately 1 mol acetate and 0.5 mol butyrate per mol crotonate. Added H₂ inhibited

growth on crotonate. Axenic cultures did not grow on butyrate, propionate, lactate, succinate, malate, fumarate, oxalate, isobutyrate, isovalerate, valerate, caproate, pyruvate or H₂ plus CO₂. We tested the ability of strain LYP^T to grow syntrophically on these substrates by co-inoculating strain LYP^T together with *Methanospirillum hungatei* into media with 10 mM of the test substrate. To determine whether the tested concentrations used were inhibitory, we also co-inoculated media containing 10 mM propionate plus 10 mM of various other substrates. These co-cultures grew and produced methane from crotonate, butyrate, propionate, malate and fumarate, but not from lactate, succinate, oxalate, valerate, caproate or pyruvate. Ability to grow on isobutyrate could not be determined because 10 mM isobutyrate was inhibitory.

Effect of temperature and pH on growth

Strain LYP^T and *Syntrophobacter wolinii* were both mesophiles with similar growth rates (Fig. 3, Table 2). Both organisms grew fastest at pH values near neutral with a similar range (Fig. 4), but with slightly different minimum pH values: strain LYP^T could not grow at pH values of 6.3 or below, whereas *Syntrophobacter wolinii* grew well at pH 6.1.

Effect of salinity on growth

Strain LYP^T and *Syntrophobacter wolinii* also differed in their sensitivity to NaCl. Each grew well in MS medium with 20 mM of substrate added (total Na⁺ concentration 120 mM), but the growth rate of strain LYP^T was reduced by 50% when 86 mM NaCl was added, and growth was completely inhibited when 171 mM NaCl was added (Fig. 5). *Syntrophobacter wolinii* was completely inhibited in the presence of 86 mM NaCl or KCl. However, it tolerated added sodium in MS medium with increased concentrations of sodium bicarbonate (Fig. 5), suggesting that it was particularly sensitive to chloride ion.

Phylogeny

The 16S rDNA sequence of strain LYP^T was determined and compared with others in the Ribosomal Database Project (RDP) and GenBank (Table 2), and Fig. 6 shows a phylogenetic tree derived from these sequences. This figure shows that strain LYP^T is most closely related (99.4% sequence similarity) to strain Syn7, a non-axenic strain of propionate-degrading methanogenic culture whose sequence was determined in the laboratory of Alfons Stams. These two strains are most closely related to *Syntrophus gentianae* (88.9% sequence similarity) and *Syntrophus buswellii* (87.8%), but these similarities were not sufficient to indicate that strain LYP^T should be classified within the genus *Syntrophobacter*. Strain LYP^T was even less related to *Syntrophobacter*, the genus of syntrophic propionate-degrading bacteria (81.1–83.0% sequence similarity).

Table 2. Sources of strains and 16S rRNA sequences

Taxon	Strain name	Culture collection*	16S rRNA sequence	Database	Reference
<i>Bdellovibrio bacteriovorus</i>	109J†	DSM 3243	M59297	RDP	Unpublished
<i>Desulfobacter postgateii</i>	11070‡	DSM 684	M26633	RDP	Pfennig & Biebl (1976)
<i>Desulfobacterium autotrophicum</i>	HRM2 ^T	DSM 3382 ^T	M34409	RDP	Devereux <i>et al.</i> (1989)
<i>Desulfomonile tiedjei</i>	DCB-1 ^T	ATCC 49306 ^T	M26635	RDP	Unpublished
<i>Desulforhabdus amnigenus</i>	ASRB1 ^T	DSM 10338 ^T	X83274	GenBank	Oude Elferink <i>et al.</i> (1995)
<i>Desulfovibrio baarsii</i>	Konstanz ^T	DSM 2075 ^T	M34403	RDP	Devereux <i>et al.</i> (1989)
<i>Desulfovibrio desulfuricans</i>	MB	ATCC 27774	M34113	RDP	Oyaizu & Woese (1985)
<i>Desulfuromonas</i> §			M80618	RDP	Amann <i>et al.</i> (1992)
<i>Desulfuromonas acetoxidans</i>	Dangast (2ac9) ^T	DSM 2034 ^T	M26634	RDP	Widdel & Pfennig (1981)
<i>Escherichia coli</i>	K-12		M29364	RDP	Shen <i>et al.</i> (1982)
' <i>Geobacter chapellei</i> '			U41561	GenBank	Loneragan <i>et al.</i> (1996)
' <i>Geobacter hydrogenophilus</i> '	H4		U46860	GenBank	Loneragan <i>et al.</i> (1996)
' <i>Geobacter hydrogenophilus</i> '	H2		U28173	GenBank	Loneragan <i>et al.</i> (1996)
<i>Geobacter metallireducens</i>	GS-15 ^T	OCM 645 ^T	L07834	GenBank	Lovley <i>et al.</i> (1993)
<i>Geobacter sulfurreducens</i>	PCA ^T		U13928	GenBank	Caccavo <i>et al.</i> (1994)
<i>Pelobacter acetylenicus</i>	WoAcy1 ^T	DSM 3246 ^T ¶	X70955	RDP	Evers <i>et al.</i> (1993)
<i>Pelobacter propionicus</i>	OttBd1 ^T	DSM 2379 ^T	X70954	RDP	Evers <i>et al.</i> (1993)
<i>Syntrophobacter</i> sp.	MPOB		X82874	GenBank	Harmsen <i>et al.</i> (1995)
<i>Syntrophobacter pfennigii</i>	KoProp1 ^T	DSM 10092 ^T	X82875	GenBank	Harmsen <i>et al.</i> (1995)
<i>Smithella propionica</i>	LYP ^T	OCM 661 ^T	X00000	GenBank	This paper
<i>Syntrophobacter wolinii</i>	(Highly purified)		X70906	GenBank	Harmsen <i>et al.</i> (1993)
<i>Syntrophobacter wolinii</i>	DB ^T	DSM 2805 ^T	X70905	RDP	Harmsen <i>et al.</i> (1993)
<i>Syntrophomonas wolfeii</i>	Göttingen ^T	OCM 41‡	M26492	GenBank	Zhao <i>et al.</i> (1989)
<i>Syntrophus buswellii</i>	(Unnamed) ^T	DSM 2612 ^T	X85131	GenBank	Wallrabenstein <i>et al.</i> (1995a)
<i>Syntrophus gentianae</i>	HQGö1 ^T	DSM 8423 ^T	X85132	GenBank	Wallrabenstein <i>et al.</i> (1995a)
	Syn7		X87269	GenBank	H. J. M. Harmsen (unpublished)

* Culture collection indicated as the source of the culture for sequencing; ATCC, American Type Culture Collection; DSM, DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen; OCM, Oregon Collection of Methanogens.

† The sequence obtained from RDP listed strain 109J as the source, but the GenBank entry for this sequence (M59297) lists the source as *Bdellovibrio bacteriovorus*, strain DSM 3243. However, DSM 3243, according to the DSMZ database, is *Methanohalophilus portucalensis* strain SF1. Our analysis of this sequence (Fig. 6) suggests that it is indeed that of *Bdellovibrio bacteriovorus*.

‡ The sequence obtained from RDP listed strain 2AC9 as the source, but the GenBank database indicates that sequence M26633 is *Desulfobacter postgateii*, strain DSM 684. DSMZ lists strain DSM 684 as the type strain of *Desulfuromonas acetoxidans* Pfennig and Biebl 1977^{AL}.

§ The GenBank database indicates that sequence M80618 is the sequence of a *Desulfuromonas*-like organism, called 'population type 2'. However, the submitters of that sequence, obtained from a methanogenic biofilm, indicate that 'population type 2' is related to *Desulfovibrio vulgaris* and 'population type 1' is related to *Desulfuromonas acetoxidans* (Amann *et al.*, 1992). Our tree places this sequence closer to that of *Desulfuromonas acetoxidans* than to those of *Desulfovibrio baarsii* and *Desulfovibrio desulfuricans*, suggesting that sequence M80618 is actually 'population type 1' described by Amann *et al.* (1992).

|| No collection was indicated in the GenBank database, but the strain is available from this collection.

¶ Evers *et al.* (1993) indicate this strain (WoAcy1) is DSM 2348, but the DSMZ database indicates that strain WoAcy1 is DSM 3246.

DISCUSSION

The membranous inclusions found in fumarate-grown *Syntrophobacter wolinii* are reminiscent of those observed in fumarate-grown *E. coli* (Lemire *et al.*, 1983; Weiner *et al.*, 1984). These structure–function studies indicated that the rod-shaped inclusions in *E. coli* had 4 nm diameter globular projections of fumarate reductase resting on a short stalk and attached to a transmembrane tube-like basal piece. Since we found membrane-delimited inclusions in *Syntrophobacter wolinii* grown on fumarate but not in those grown on

pyruvate, it is possible that these inclusions also were sites of fumarate reductase activity.

Syntrophobacter wolinii degrades propionate with a stoichiometry of 1 mol acetate formed per mol propionate degraded, whether this degradation is coupled syntrophically to sulfate reduction or to methanogenesis (Boone & Bryant, 1980). This stoichiometry (Table 1, equation A) is consistent with the randomizing pathway that has been identified in this bacterium (Houwen *et al.*, 1990). Although the amount of H₂ or formate was not directly measured, its

Fig. 4. Effect of pH on growth rates of *Smithella propionica* LYP^T (□) and *Syntrophobacter wolinii* DB^T (○).

Fig. 5. Effect of added salts on growth rates of *Smithella propionica* LYP^T and *Syntrophobacter wolinii* DB^T. *Syntrophobacter wolinii* with sodium chloride (●) or sodium bicarbonate (○) added to MS medium; *Smithella propionica* LYP^T (□).

calculated value (calculated stoichiometrically from the ultimate product, H₂S or CH₄) was near the expected amount. In contrast, strain LYP^T in co-culture with *Methanospirillum hungatei* formed much smaller amounts of methane and larger amounts of acetate during propionate degradation, with a stoichiometry closer to equations F+H (Table 1). These deviations from expected stoichiometry could be explained by acetogenesis from CO₂ and H₂, but strain LYP^T was unable to grow on H₂ plus CO₂. Another possibility involves the reductive carboxylation of propionate to butyrate or butyryl-CoA, which has been documented in anaerobic digestors (Tholozan *et al.*, 1988). This latter possibility is supported by the ability of strain LYP^T to grow slowly on crotonate, and to grow in syntrophic co-culture on butyrate. These abilities indicate that it can couple energy to the β -oxidation of butyrate, as has been shown for *Syntrophomonas wolfeii* (McInerney *et al.*, 1979, 1981; McInerney & Wofford, 1992). This finding, together with the stoichiometry of its syntrophic degradation of propionate, is consistent with the dismutation of propionate to acetate and butyryl-CoA, followed by syntrophic β -oxidation of butyryl-CoA to acetate. The presence of a kinase or HS-CoA transferase of low activity would explain the release of small quantities of butyrate from butyryl-CoA, and this would also allow the observed slow growth of strain LYP^T on butyrate (syntrophically) or on crotonate. This pathway is also consistent with the thermodynamics of these reactions, which were always negative during the batch growth of co-cultures (Fig. 2c).

The production of butyrate during syntrophic propionate degradation distinguished *Smithella propionica* from *Syntrophobacter wolinii*. We confirmed the previous finding (Boone & Bryant, 1980) that *Syntrophobacter wolinii* also does not produce butyrate from propionate; the production of small amounts of butyrate might not have been detected in that study because the rumen-fluid medium used in the original study contained small amounts of butyrate.

Taxonomy

Our findings indicate that strain LYP^T differs morphologically, physiologically and phylogenetically from previously described propionate-degrading bacteria. Strain LYP^T is phylogenetically most similar to the genus *Syntrophus*, but the phylogenetic relationship is not sufficiently close to permit classification of strain LYP^T in this genus. Also, the ability of strain LYP^T to grow on propionate separates it phenotypically from members of this genus. Therefore we propose a new genus and species, *Smithella propionica* gen. nov., sp. nov.

Description of *Smithella* gen. nov.

Smithella (Smi.thel'la. N.L. n. *Smithella* Smith, named after Paul H. Smith, for his early contributions to the

Fig. 6. Phylogenetic tree comparing *Smithella propionica* LYP^T with other bacteria, based on a distance-matrix analysis of an approximately 683 base segment of the 16S rDNA and rRNA sequences. These data compared by parsimony and maximum-likelihood methods gave trees similar to this one. A partial tree is shown, in which *E. coli* was used as the outgroup. Bar, 5 base substitutions per 100 bases.

understanding of propionate degradation in methanogenic ecosystems).

Weakly motile Gram-negative rods. Strictly anaerobic. Mesophilic. Growth by syntrophic catabolism of propionate involving its reductive carboxylation to butyrate or butyryl-CoA. Habitat: methanogenic environments in which propionate is degraded. Type species: *Smithella propionica*.

Smithella propionica (pro.pi.o'ni.ca. M.L. n. *acidum propionicum* propionic acid; M.L. adj. *propionica* pertaining to propionic acid, on which the bacterium grows).

Gram-negative rods, 0.5 µm in diameter, with most cells 3–5 µm long, but some cells as long as 10 µm. Contain granules of poly-β-hydroxybutyrate. Weakly motile. Strictly anaerobic. Mesophilic. Fastest growth

with pH near neutral and Na⁺ and Cl⁻ concentrations less than 100 mM. Grows syntrophically on propionate with H₂- or formate-using methanogenic partner. Grows slowly axenically by dismutating crotonate to butyrate and acetate. Habitat: methanogenic digestors. Type strain is strain LYP^T (= OCM 661^T).

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