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 digestor and isolated. Initial isolation was as a monoxenic propionate-degrading co-culture containing Methanospirillum hungateii JF-1\(^T\) as an \(H_2\)- 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 hungateii, forming acetate, \(CO_2\) 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 digestor

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 \(CO_2\) (Boone, 1984; Stadtman & Barker, 1951), with the electrons generated from this oxidation ultimately transferred to methanogens that form methane by \(CO_2\) reduction. \(H_2\) (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 \(H_2\) and formate production as the electron sink is exergonic only when \(H_2\) 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...
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 wolini* 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 wolini* from its co-culture with *Desulfovibrio G11* in monoxenic co-culture with *Methanospirillum hungateii* and in axenic culture with fumarate as a catabolic substrate.

Propionate degradation by *Syntrophobacter wolini* (Houwen et al., 1991), anaerobic sediments (Schink, 1985), anaerobic digesters (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 digesters 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 digesters. 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, 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 digesters (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 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 wolini* DB7 in co-culture with *Desulfovibrio G11* (= OCM 467), *Syntrophomonas wolfeii* LYP (= OCM 65), which is a butyrate-oxidizing syntroph (Boone et al., 1989). *Methanosarcina barkeri* NIH-1 (= OCM 266) was purified from *Methano- sarcina barkeri* NIH (= OCM 265) (Blaylock & Stadtman, 1966) by roll-tube isolation in MS medium with 50 mM methanol. *Methanospirillum hungateii* JF17 (= OCM 165*) 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). Excep where noted, we used MS medium (Boone et al., 1989), an

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

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Equation</th>
<th>ΔG° (kJ/reaction)†</th>
<th>H₂ at equilibrium*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Hydrogen-producing reactions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>CH₃CH₂COO⁻ + 2H₂O → CH₃COO⁻ + CO₂ + 3H₂</td>
<td>71.7</td>
<td>12.1 89.2</td>
</tr>
<tr>
<td>B</td>
<td>CH₃CH₂CH₂COO⁻ + 2H₂O → 2CH₃COO⁻ + H⁺ + 2H₂</td>
<td>48.3</td>
<td>275 2024</td>
</tr>
<tr>
<td><strong>II. Hydrogen-consuming reactions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>CO₂ + 4H₂ → CH₄ + 2H₂O</td>
<td>-130.7</td>
<td>0.317 2.33</td>
</tr>
<tr>
<td>D</td>
<td>CH₃CH₂COO⁻ + CO₂ + 3H₂ → CH₃CH₂CH₂COO⁻ + 2H₂O</td>
<td>-71.5</td>
<td>12.3 90.6</td>
</tr>
<tr>
<td>E(D + B)</td>
<td>CH₃CH₂COO⁻ + CO₂ + H₂ → 2CH₃COO⁻ + H⁺</td>
<td>-23.3</td>
<td>0.025 0.18</td>
</tr>
<tr>
<td><strong>III. Combined hydrogen-producing and hydrogen-consuming reactions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F(B + 1/2C)</td>
<td>CH₃CH₂CH₂COO⁻ + 1/2CO₂ + H₂O → 2CH₃COO⁻ + H⁺ + 1/2H₂</td>
<td>-17.1</td>
<td></td>
</tr>
<tr>
<td>G(4A + 3C)</td>
<td>4CH₃CH₂COO⁻ + 2H₂O → 4CH₃COO⁻ + CO₂ + 3CH₄</td>
<td>-105.6</td>
<td></td>
</tr>
<tr>
<td>H(A + D)</td>
<td>2CH₃CH₂COO⁻ → CH₃COO⁻ + CH₃CH₂CH₂COO⁻</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>I(3F + A)</td>
<td>4CH₃CH₂COO⁻ + 2CO₂ + 2H₂O → 7CH₃COO⁻ + 3H⁺</td>
<td>1.91</td>
<td></td>
</tr>
</tbody>
</table>

* H₂ activity for ΔG° = 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 10⁻⁵ mol activity per kg; all other substances in aqueous solution at 1 mol of activity per kg; 25 °C (Thauer et al., 1977).
anaerobic bicarbonate-buffered medium with 2 g l$^{-1}$ each of Trypticase peptone and yeast extract; MES (0.5 g l$^{-1}$) and Na$_2$S. 9H$_2$O (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 15 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 (N$_2$) 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 cultures was monitored by optical density at 670 nm (Spectronic 21; Milton Roy).

**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 aqueous safranin, and stained for detection of poly-P-hydroxyalkanoate (Doetsch, 1980).

**Cytochemical stain for poly-hydroxyalkanoate.** Cells were stained for detection of poly-$\beta$-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 glucose-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 & Fritsch, 1989). The PCR amplification primers (Weisburg et al., 1991) were F1 (5' AGAGTTTGATCCTGGCTCAG) and rP2 (5' AGCGCT-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' ACGGCGGTTGTGATAC) (Lane et al., 1985), corresponding to positions 1406-1392 in the 16S rDNA nucleotide sequence of Escherichia coli (Brosius et al., 1978); G-complement (5' GTACACACC-CCGT; E. coli positions 1392-1406); H (5' ACACGA- GCTGACGACGACCA; positions 1075-1056); G (5' CCA-GGTTATCTAATCTCGT; positions 800-781); G-complement (5' AACAAGATTCGTTACGTTCTG; positions 781-800); A (5' GTATTACCCGGGC/CTGTCG; positions 536-519); P (5' CTGCTTATCCTGGAGGAG; positions 357-339); P-complement (5' CTACGGGAGGC- AGCAG; positions 342-357); and F (5' AGAGTT- TGATC(A/C)TGTCCT; 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 hungateii*. These dilutions were also inoculated into solidified medium in roll tubes with lawns of *Methanospirillum hungateii*, 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 Methanospirillurn hungateii 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 Methanospirillurn hungateii 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 Methanospirillurn hungateii 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 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 and deposited in OCM as OCM 661T. 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 were successfully applied to Syntrophobacter wolinii. We grew cultures of the triculture of strain LYP, Desulfovibrio strain G11 and Methanospirillurn hungateii (Boone & Bryant, 1980) in MS mineral medium plus 20 mM propionate. After six transfers, Methanospirillurn hungateii and Syntrophobacter wolinii were dominant, with very few cells of Desulfovibrio strain G11 evident. We inoculated dilutions of this culture into MS medium with propionate as substrate, also adding 0-1 nl of a culture of Methanospirillurn hungateii 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 Methanospirillurn hungateii and of Syntrophobacter wolinii. This co-culture was inoculated into roll-tube medium with propionate and lawns of both Methanospirillurn hungateii and Methanosarcina barkeri. After 6 months incubation, light yellow colonies formed that comprised three morphological types, attributed to Methanospirillurn hungateii, 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 and Methanospirillurn hungateii 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 Methanospirillurn hungateii, 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. This culture was deposited in OCM as OCM 587T. It grew on malate, fumarate or pyruvate, but not on propionate. However, when co-inoculated with Methanospirillurn hungateii into MS medium with 20 mM propionate, the culture grew and formed methane in expected amounts.

Morphology

Strain LYP 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
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<sup>T</sup> co-cultures from axenic cultures**

Like *Syntrophomonas wolfeii*, strain LYP<sup>T</sup> 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<sup>T</sup> was co-inoculated with *Methanogenium* strain PM or *Methanospirillum hungateii*, the co-cultures grew slowly on butyrate, and produced methane in expected quantities. Likewise, an axenic culture of strain LYP<sup>T</sup> did not grow in MS medium with 20 mM propionate, but it grew in this medium when co-inoculated with *Methanospirillum hungateii* 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<sup>T</sup>**

When a reconstituted co-culture of strain LYP<sup>T</sup> and *Methanospirillum hungateii* 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).
Inhibition by H₂ or formate

To determine whether inefficient H₂ (or formate) removal limited propionate oxidation by strain LYPᵀ, we increased the numbers of methanogens in the co-culture. We did this by pre-growing Methanospirillum hungateii 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 LYPᵀ 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 LYPᵀ 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 LYPᵀ on propionate depended on the removal of H₂ and formate by Methanospirillum hungateii.

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 acetilastic methanogen Methanosarcina barkeri was co-inoculated with the co-culture of strain LYPᵀ and Methanospirillum hungateii, 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-
lated in co-cultures of strain LYP$^T$ and *Methanospirillum hungateii* 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 hungateii*. 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 hungateii* and *Methanosarcina bar keri* 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 H$_2$ inhibited growth on crotonate. Axenic cultures did not grow on butyrate, propionate, lactate, succinate, malate, fumarate, oxalate, isobutyrate, isovalerate, valerate, caproate, pyruvate or H$_2$ plus CO$_2$. We tested the ability of strain LYP$^T$ to grow syntrophically on these substrates by co-inoculating strain LYP$^T$ together with *Methanospirillum hungateii* 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). 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).
Y. Liu and others

Table 2. Sources of strains and 16S rRNA sequences

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Strain name</th>
<th>Culture collection*</th>
<th>16S rRNA sequence</th>
<th>Database</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Bdellovibrio bacteriovorus</em></td>
<td>DSM 3243</td>
<td>M59297</td>
<td>RDP</td>
<td>Unpublished</td>
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<td>Desulfo bacter postgateii</td>
<td>DSM 684</td>
<td>M26633</td>
<td>RDP</td>
<td>Pfennig &amp; Biel (1976)</td>
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<td>Desulfomonile tigdjeii</td>
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<td>M26635</td>
<td>RDP</td>
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<td>Desulfobacteriobusammigenus</td>
<td>DSM 10338T</td>
<td>X83274</td>
<td>GenBank</td>
<td>Oude Elferink et al. (1995)</td>
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<td>Desulfovibrio baarsii</td>
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<td>M34403</td>
<td>RDP</td>
<td>Devereux et al. (1989)</td>
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<td>Desulfovibrio desulfuricans</td>
<td>DSM 27774</td>
<td>M34113</td>
<td>RDP</td>
<td>Oyazi &amp; Woese (1985)</td>
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<tr>
<td>Desulfuromonas acetoxydans</td>
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<td>M26634</td>
<td>RDP</td>
<td>Widdel &amp; Pfennig (1981)</td>
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<tr>
<td>Escherichia coli</td>
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<td>RDP</td>
<td>Shen et al. (1982)</td>
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<td>‘Geobacter chapellei’</td>
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<td>GenBank</td>
<td>Lonergan et al.</td>
<td>(1996)</td>
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<td>Lovey et al.</td>
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<td>Caccavo et al.</td>
<td>(1994)</td>
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<td>Evers et al. (1993)</td>
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<td>X82874</td>
<td>GenBank</td>
<td>Harmsen et al.</td>
<td>(1995)</td>
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<td>Harmsen et al.</td>
<td>(1995)</td>
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<td>Harmsen et al.</td>
<td>(1993)</td>
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<td>X00000</td>
<td>GenBank</td>
<td>This paper</td>
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<td>Syntrophobacter wolinii</td>
<td>(Highly purified)</td>
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<td></td>
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<td>DBT</td>
<td>DSM 2805T</td>
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<td>(1993)</td>
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<td>Zhao et al.</td>
<td>(1989)</td>
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<td>Wallrabenstein et al. (1995a)</td>
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<tr>
<td>Synthrophus gentianeae</td>
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<td>X85132</td>
<td>GenBank</td>
<td>Wallrabenstein et al. (1995a)</td>
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<tr>
<td>Synthrophobacter wolinii</td>
<td>Syn7</td>
<td>X87269</td>
<td>H. J. M. Harmsen (unpublished)</td>
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</tbody>
</table>

*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 *Desulfovibrio postgateii*, strain DSM 684. DSMZ lists strain DSM 684 as the type strain of *Desulfovibrio postgateii*; strain DSM 684. DSMZ lists strain DSM 684 as the type strain of *Desulfovibrio desulfuricans*.

§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 acetoxydans* (Amann et al., 1992). Our tree places this sequence closer to that of *Desulfuromonas acetoxydans* 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.

| Remarks |

4 Even the accession number, a genome accession sequence closer to that of *Desulfovibrio vulgaris*, indicates that ‘population type 2’ is related to *Desulfovibrio vulgaris* and ‘population type 1’ is related to *Desulfuromonas acetoxydans*. The sequence obtained from RDP listed strain 2AC9 as the source, but the GenBank database indicates that sequence M26633 is *Desulfovibrio postgateii*, strain DSM 684. DSMZ lists strain DSM 684 as the type strain of *Desulfovibrio postgateii*; strain DSM 684. DSMZ lists strain DSM 684 as the type strain of *Desulfovibrio desulfuricans*.

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...
calculated value (calculated stoichiometrically from the ultimate product, \( \text{H}_2\text{S} \) or \( \text{CH}_4 \)) was near the expected amount. In contrast, strain \( \text{LYP}^T \) in co-culture with \( \text{Methanospirillum hungatei} \) 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 \( \text{CO}_2 \) and \( \text{H}_2 \), but strain \( \text{LYP}^T \) was unable to grow on \( \text{H}_2 \) plus \( \text{CO}_2 \). 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 \( \text{LYP}^T \) to grow slowly on crotonate, and to grow in syntrophic co-culture on butyrate. These abilities indicate that it can couple energy to the \( \beta \)-oxidation of butyrate, as has been shown for \( \text{Syntrophomonas wolfei} \) (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 \( \beta \)-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 \( \text{LYP}^T \) 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 \( \text{Smithella propionica} \) from \( \text{Syntrophobacter wolinii} \). We confirmed the previous finding (Boone & Bryant, 1980) that \( \text{Syntrophobacter wolinii} \) 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 \( \text{LYP}^T \) differs morphologically, physiologically and phylogenetically from previously described propionate-degrading bacteria. Strain \( \text{LYP}^T \) is phylogenetically most similar to the genus \( \text{Syntrophus} \), but the phylogenetic relationship is not sufficiently close to permit classification of strain \( \text{LYP}^T \) in this genus. Also, the ability of strain \( \text{LYP}^T \) to grow on propionate separates it phenotypically from members of this genus. Therefore we propose a new genus and species, \( \text{Smithella propionica} \) gen. nov., sp. nov.

**Description of \text{Smithella} gen. nov.**

\( \text{Smithella} \) (Sm.i.thel'la. N.L. n. \textit{Smithella} Smith, named after Paul H. Smith, for his early contributions to the
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, 0.5-5 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 LYP2 (= OCM 661T).

We thank Shuisong Ni (Washington State University, Richland) for helpful discussions, Donna S. Williams for help with the electron microscopy, and Sylvia E. Coleman for determining the presence of poly-β-hydroxyalkanoate granules. This work was supported by grant DE-FG05-90ER61039 from the US Department of Energy through the Subsurface Science Program (Deep Microbiology Subprogram) and by a subcontract from master contract 206010 (to Pacific Northwest National Laboratory), task order 258705.

Fig. 6. Phylogenetic tree comparing Smithella propionica LYP2 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.
REFERENCES


