The first true obligately syntrophic propionate-oxidizing bacterium, *Pelotomaculum schinkii* sp. nov., co-cultured with *Methanospirillum hungatei*, and emended description of the genus *Pelotomaculum*

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A Gram-positive, spore-forming, syntrophic propionate-oxidizing bacterium, *Pelotomaculum schinkii* sp. nov. strain HH^T, was isolated as a co-culture with *Methanospirillum hungatei* JF-1^T from anaerobic, freeze-dried granular sludge obtained from an upflow anaerobic sludge bed reactor treating sugar beet wastewater. The bacterium converted propionate to acetate in co-culture with *Methanospirillum hungatei* JF-1^T or *Methanobacterium formicicum* MF^{NT}, but not in co-culture with *Methanobrevibacter arboriphilus* AZ. The organism could not be cultured axenically with any of the substrates tested and therefore can be considered as a (the first) true anaerobic syntrophic bacterium. The bacterium contained two distinct 16S rRNA gene sequences, with 96·8 % sequence similarity, which were both expressed during syntrophic growth on propionate as revealed by fluorescent *in situ* hybridization. The most closely related organisms are *Cryptanaerobacter phenolicus* LR7.2^T, a bacterium that transforms phenol into benzoate, and *Pelotomaculum thermopropionicum* SI^T, a thermophilic, syntrophic propionate-oxidizing bacterium. Other related species belong to the Gram-positive, sulfate-reducing genus *Desulfotomaculum*. The type strain of *Pelotomaculum schinkii* is strain HH^T (=ATCC BAA-615^T=DSM 15200^T).

In methanogenic habitats, complex organic matter is degraded completely to CO_2 and CH_4 (Bryant, 1976). Propionate oxidation is an important step in this process, which requires obligately syntrophic consortia of acetogenic bacteria and methanogenic archaea. The methanogens make propionate oxidation energetically feasible by keeping the concentrations of the products H_2 and formate extremely low (de Bok *et al.*, 2004; Schink, 1997; Schink & Stams, 2002). Most of the syntrophic propionate-oxidizing bacteria isolated so far belong to the *Syntrophobacter* cluster within the '*Deltaproteobacteria*'. *Syntrophobacter* species are able to use sulfate as the electron acceptor for propionate oxidation (Harmsen *et al.*, 1993; Wallrabenstein *et al.*, 1995). In addition, they can grow by fermentation of pyruvate and fumarate. *Smithella propionica* LYP^T, which was isolated more recently, is phylogenetically related to the genus *Syntrophus* (Liu *et al.*, 1999). This Gram-negative propionate-oxidizer lacks the ability to reduce sulfate and uses a different pathway to oxidize propionate from that used by *Syntrophobacter* strains. However, *Smithella propionica* LYP^T is able to grow axenically on crotonate (de Bok *et al.*, 2001; Liu *et al.*, 1999). Syntrophic propionate oxidation is not restricted to Gram-negative bacteria. Wu *et al.* (1992) described a Gram-positive, syntrophic propionate-oxidizing bacterium that is able to produce spores. Apart from its grouping within the Gram-positive bacteria, no further physiological characterization was reported. Two thermophilic, Gram-positive, syntrophic propionate-oxidizing bacteria have been described, *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* TPO^T, which grows axenically on several substrates and, like *Syntrophobacter* strains, can use sulfate as an electron acceptor

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Abbreviation: FISH, fluorescent *in situ* hybridization.

The GenBank/EMBL/DDBJ accession numbers for the 'spore A' and 'spore B' 16S rRNA gene sequences of strain HH^T are respectively X91169 and X91170.

(Plugge *et al.*, 2002), and *Pelotomaculum thermopropionicum* SI^T, which lacks the ability to reduce sulfate but ferments pyruvate and fumarate in pure culture (Imachi *et al.*, 2000, 2002). In this study, we describe a mesophilic, spore-forming, syntrophic propionate-oxidizing bacterium, strain HH^T, which is also closely related to *Pelotomaculum thermopropionicum* SI^T and the genus *Desulfotomaculum*. This strain was obtained as a defined co-culture with *Methanospirillum hungatei* JF-1^T and could not grow axenically on any of the substrates tested.

Methanospirillum hungatei JF-1^T (=DSM 864^T), *Methanobacterium formicicum* MF^{NT} (=DSM 1535^{NT}) and *Methanobrevibacter arboriphilus* AZ (=DSM 744) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). A bicarbonate-buffered mineral medium was used, with the following composition: 3 mM Na_2HPO_4, 3 mM KH_2PO_4, 5·6 mM NH_4Cl, 0·75 mM $CaCl_2$, 0·5 mM $MgCl_2$, 5 mM NaCl, 50 mM $NaHCO_3$, 1 mM Na_2S, 7·5 μM $FeCl_2$, 1 μM H_3BO_3, 0·5 μM $ZnCl_2$, 0·1 μM $CuCl_2$, 0·5 μM $MnCl_2$, 0·5 μM $CoCl_2$, 0·1 μM $NiCl_2$, 0·1 μM Na_2SeO_3, 0·1 μM Na_2WO_4, 0·1 μM Na_2MoO_4, 0·5 mg EDTA l^{-1} and the following vitamins (mg l^{-1}): 0·02 biotin, 0·2 nicotinic acid, 0·5 pyridoxine, 0·1 riboflavin, 0·2 thiamin, 0·1 cyanocobalamin, 0·1 *p*-aminobenzoic acid, 0·1 pantothenic acid, 0·1 lipoic acid and 0·1 folic acid. Methanogenic archaea were cultured routinely at 37 °C in 120 ml serum flasks with 50 ml medium and a gas phase of 1·7 atm. H_2/CO_2 (80 : 20, v/v). Prior to inoculation of the propionate-oxidizing bacteria, CH_4 and residual H_2 were removed and replaced by 1·7 atm. N_2/CO_2 (80 : 20, v/v). Propionate-oxidizing bacteria were inoculated in H_2/CO_2-pregrown cultures of *Methanospirillum hungatei* JF-1^T, *Methanobacterium formicicum* MF^{NT} or *Methanobrevibacter arboriphilus* AZ. Routinely, 5 % of a pasteurized (20 min, 85 °C) methanogenic co-culture was used as the inoculum. After addition of sodium propionate (20 mM final concentration), the cultures were incubated at 37 °C. Percoll density-gradient centrifugation was carried out under anoxic conditions using a glove box with N_2/H_2 (96 : 4, v/v) as the gas phase and airtight centrifuge tubes for centrifugation steps outside the glove box. Traces of oxygen were removed from the glove box by circulating the gas phase over a platinum catalyst column. Cells were collected from a 0·5 l propionate-oxidizing enrichment culture by centrifugation at 16 000 ***g***. The cell pellet was resuspended in a mixture of 70 % Percoll and 50 mM sodium phosphate, pH 7·5, containing 2 mM Na_2S. The cells were separated in a Percoll gradient, which was generated in a 9 ml centrifuge tube at 30 000 ***g*** and 18 °C for 30 min.

Organic acids were measured with a Spectrasystem HPLC system equipped with an autosampler and refractometer. The acids were separated on a Polyspher OAHY column (30 cm × 6·5 mm; Merck) in 0·005 M H_2SO_4 at a flow rate of 0·6 ml min^{-1} and a column temperature of 60 °C. The acids eluting from the column were quantified by differential refractometry. H_2 and CH_4 were measured using a Packard-Becker 417 gas chromatograph equipped with a thermal conductivity detector and a molecular sieve 13X (60/80 mesh). The column temperature was 50 °C and the carrier gas was argon at a flow rate of 30 ml min^{-1}. To determine whether strain HH^T uses a randomizing pathway of propionate oxidation, a co-culture was grown in the presence of 5 mM 3-^{13}C-propionate (de Bok *et al.*, 2001). At the end of the exponential growth phase, a sample was withdrawn from this culture and analysed for isotopes using ^{13}C-NMR spectroscopy. A proton-decoupled ^{13}C-NMR spectrum was recorded at 75·47 MHz on a Bruker AMX-300 NMR spectrometer as described previously (de Bok *et al.*, 2001).

Nucleic acids were isolated from 10 ml of a late-exponential-phase enrichment culture as described previously (Harmsen *et al.*, 1995). Bacterial 16S rRNA genes present in the enrichment culture were amplified by PCR as described by Harmsen *et al.* (1993) using a set of universal 16S rRNA-based primers containing restriction sites (in bold) for *Bam*HI and *Pst*I to facilitate cloning: the forward primer (5′-CAC**GGATCC**GGACGGGTGAGTAACACG) corresponded to *Escherichia coli* positions 106–124 and the reverse primer (5′-GTG**CTGCAG**GGTTACCTTGTTAC GACT) to *E. coli* positions 1493–1510. Recombinant plasmids were obtained as described previously using pUC18 as cloning vector and *E. coli* TG1 as host (Harmsen *et al.*, 1996). DNA sequencing was done using the dideoxy chain-termination method (Sanger *et al.*, 1977) adapted for cycle-sequencing with *Taq* polymerase as described in the Life Science Technologies (now Invitrogen) manual. All enzymes for DNA manipulations were obtained from Life Science Technologies. Unlabelled oligonucleotides were purchased from Pharmacia. Partial 16S rRNA gene nucleotide sequences were aligned with those of other bacteria, taking into account sequence similarity and higher order structure, using the alignment tool of the ARB software package (Strunk & Ludwig, 1995). Slot-blot hybridization experiments were performed on Hybond N+ filters (GE Healthcare). Nucleic acid samples containing approximately 50 ng DNA were applied to the membrane with a Hybridot manifold (Life Science Technologies) and immobilized according to Church & Gilbert (1984). The membranes were pretreated with hybridization buffer (0·5 M sodium phosphate, pH 7·2, 7 % SDS, 1 % BSA and 1 mM EDTA) for 30 min prior to hybridization with 100 ng [γ-^{32}P]ATP-labelled probes. All membranes were hybridized at 40 °C and washed in 1 % SDS, 1× SSC (0·15 M NaCl, 0·015 M sodium citrate, pH 7·0) wash buffer at 50 °C. The membranes were exposed to a Kodak X-ray film or exposed to a phosphorimager screen. The screen was then scanned for radioactive response on a PhosphorImager (Molecular Dynamics). Digital signals were processed by the manufacturer's software (ImageQuant). Cells were prepared for fluorescent *in situ* hybridization (FISH) either by mixing culture samples 1 : 1 directly with 8 % paraformaldehyde or by concentrating

the culture samples by centrifugation and then resuspending the cell pellets in PBS (130 mM NaCl, 10 mM sodium phosphate, pH 7·2) containing 4 % paraformaldehyde. Cells were fixed for 2 h and then washed with PBS and resuspended in PBS/ethanol (1 : 1). Alternatively, cells were fixed by mixing the culture samples 1 : 1 with ice-cold 96 % ethanol or resuspending cell pellets in PBS/ethanol (1 : 1). Samples were stored at −20 °C. Samples were dried onto glass slides (10 µl per well), washed with Milli-Q water and then dehydrated in an increasing ethanol series (3 min each in 50, 80 and 96 % v/v ethanol). To test whether lysozyme improved the permeabilization, slides were incubated in a lysozyme solution (100 mg ml^{-1}) for 10 min at 50 °C. The cells were hybridized for 1·5 h with fluorescein- or Cy3-labelled oligonucleotide probes at 50 °C and formamide at 0, 10, 20, 30, 40 and 50 %, according to Amann (1996). Fluorescent cells were detected with an Olympus BH2 epifluorescence microscope.

The propionate-oxidizing culture was enriched from freeze-dried granular sludge from a full-scale upflow anaerobic sludge bed (UASB) reactor treating sugar beet waste (CSM, Breda, The Netherlands). After freeze-drying, the sludge had been stored aerobically for more than 2 years at room temperature. Bottles with freshly prepared medium (50 ml) were inoculated with 0·2 g freeze-dried granular sludge. After addition of 20 mM sodium propionate and 10 % (v/v) of a H_2/CO_2-grown culture of *Methanospirillum hungatei* JF-1^T, the enrichment culture was incubated at 37 °C. After 3 months, the propionate was depleted and CH_4 was produced. Acetate was detected in only small amounts, indicating the presence of acetoclastic methanogens. Propionate-oxidizing bacteria were further purified by repeated rounds of pasteurization for 30 min at 85 °C and subculturing in the presence of *Methanospirillum hungatei* JF-1^T. The highest dilution with growth (usually dilution 10^8) was always used for subculturing. After six transfers, the culture consisted of two morphotypes, *Methanospirillum hungatei* JF-1^T and a rod-shaped bacterium, which formed endospores (Fig. 1). No other bacteria were observed microscopically. However, upon addition of glucose, a long-rod-shaped bacterium developed. This bacterium remained, even after additional rounds of pasteurization and subculturing. The doubling time of the culture was approximately 7 days and the culture had a lag phase varying from 1 to 8 weeks.

To isolate strain HH^T as a defined co-culture with *Methanospirillum hungatei* JF-1^T, cells from a 0·5 l propionate-oxidizing enrichment culture were separated in a Percoll gradient. Two bands were visible in the gradient, of which the upper band contained the (short) rod-shaped bacteria and the lower band *Methanospirillum hungatei*. Cells with the morphology of the long-rod-shaped bacteria were not detected (microscopically) in either of these bands. The upper band containing the propionate-oxidizing bacteria was collected and inoculated in freshly prepared basal medium. Four dilution series up to 10^{10} dilution were prepared from this culture in exponentially growing *Methanospirillum hungatei* JF-1^T cultures from which CH_4 and residual H_2 were removed by flushing with N_2/CO_2. In two of the four dilution series, yeast extract (1 g l^{-1}) and casein tryptic peptones (1 g l^{-1}) were added to stimulate growth. Sodium propionate (20 mM) was added to each of the cultures and all bottles were incubated at 37 °C in the dark. In the dilution series containing yeast extract and peptones, CH_4 was produced up to the 10^9 dilution within 6 weeks. At this time-point, CH_4 was detected only in the 10^1 dilution of the series that did not contain yeast extract and peptones. However, after 4 months, CH_4 was produced up to the 10^9 dilution in the series without yeast extract and peptones. Purity of this culture was checked by inoculating batches in medium with glucose (10 mM), pyruvate (20 mM), yeast extract (1 g l^{-1}) or casein tryptic peptones (1 g l^{-1}). Growth was not observed in any of these cultures, indicating that the culture consisted of the spore-forming, propionate-oxidizing bacterium and *Methanospirillum hungatei* JF-1^T only (Fig. 1). The propionate-oxidizing bacteria were 1 × 2·0–2·5 µm in size; non-motile and produced centrally located endospores in the late exponential phase. Sequence analysis of the 16S rRNA genes amplified from this culture revealed that both sequences obtained from the enrichment culture were still present in equal amounts in this culture.

An enrichment culture that had been pasteurized and subcultured at least 15 times under syntrophic conditions was used for phylogenetic analysis. PCR amplification of the 16S rRNA genes from nucleic acids isolated from this culture resulted in a product of the expected size of 1·4 kb. This fragment was digested with *Bam*HI and *Pst*I and ligated in pUC18 linearized with the same enzymes. The ligation products were transformed into *E. coli* TG1 cells, which resulted in 38 recombinant plasmids. Ten of the recombinant plasmids were characterized by sequence analysis of the insert DNA using the universal 16S rRNA primer 1115 (*E. coli* positions 1100–1115; Lane, 1991). Four of these plasmids contained inserts with identical sequences of the V6 region of the 16S rRNA, indicated as 'spore A' sequences. Five other plasmids also contained identical sequences, indicated as 'spore B'. One plasmid contained an unidentified sequence. Two plasmids containing a spore A or B 16S rRNA gene sequence were selected for further analysis and their inserts were sequenced completely, resulting in sequences of 1361 and 1362 bp, respectively. These sequences have been deposited in the EMBL database with accession numbers X91169 (spore A) and X91170 (spore B). The similarity between the two 16S rRNA gene sequences was 96·8 %. Specific oligonucleotide probes were designed against the V6 region of the type A and type B 16S rRNA gene sequences (5′-GGACTACTGACACCTTTGTGTCTC and 5′-GAGACTGTCGGATACTTTCATCC for A and B, respectively). A slot blot containing approximately 100 ng of each of the 38 plasmids was hybridized with both probes to determine the ratio in which the clone library represented the two sequence types. In addition, a comparable amount

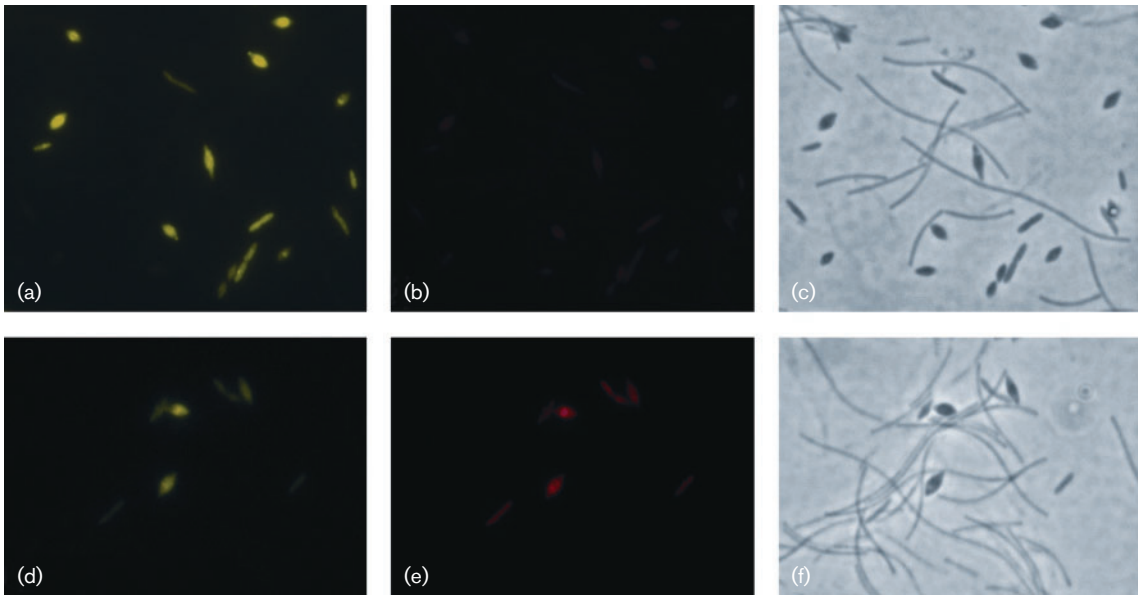

Fig. 1. *In situ* hybridization of the spore-forming, syntrophic propionate-oxidizing culture. (a) Cells probed with the fluorescein-labelled universal probe Eub338. (b) Background fluorescence through a Cy3 filter in the same field as (a) without Cy3 probe. (d)–(e) Cells probed with fluorescein-labelled spore A-specific probe SPA177 (d) and the Cy3-labelled spore B-specific probe SPB177 (e). Corresponding phase-contrast micrographs are shown in (c) and (f). The long, thin, curved rods are *Methanospirillum hungatei* JF-1^T; all other cells belong to strain HH^T.

of DNA obtained from five different PCR amplifications was added to the blot to determine whether selective cloning had occurred. Thirteen of the 38 plasmids gave a positive signal with the type A-specific probe and 17 plasmids gave a positive signal with the type B-specific probe. All five PCR products hybridized with both probes with approximately the same efficiency, indicating that the two 16S rRNA gene sequences were present in equal amounts after amplification.

Sequence heterogeneities in the rRNA operons are not an unusual property of bacteria, as this has also been described for other Gram-positive bacteria such as *Paenibacillus polymyxa* (Nubel *et al.*, 1996), *Bifidobacterium adolescentis* (Satokari *et al.*, 2001) and *Clostridium perfringens* (Shimizu *et al.*, 2001). However, to exclude the possibility that there were two closely related bacterial species left in the co-culture, FISH was used to target both sequences in individual cells. Based on sequence differences observed in the V2 regions of the 16S rRNA gene sequences (Table 1), two fluorescently labelled oligonucleotide probes were designed to determine whether the type A and type B sequences belonged to a single organism. The best hybridization results were obtained with early exponential-phase cells that were fixed with paraformaldehyde and hybridized in the presence of 10 % formamide. Both V2 probes were detected in the same cells and none of the cells hybridized with only one of these probes, although faint signals were detected in some cells (Fig. 1). To exclude cross-hybridization with non-target bacteria, three environmental strains, *Succiniclasticum ruminis* DSM 9236^T, *Sporomusa paucivorans* DSM 3697^T and *Clostridium sporosphaeroides* DSM 1294^T, and five clinical strains (identified as *Eubacterium cylindroides*, *Clostridium butyricum*, *Clostridium nexile*, *Fusobacterium mortiferum* and *Clostridium cadaveris*) were fixed and hybridized with both V2 probes under the conditions which were optimal for strain HH^T, but a signal was detected for none of these strains. Based on these results, we conclude that strain HH^T contains two different 16S rRNA gene sequences which are both expressed during syntrophic growth on propionate.

The propionate-oxidizing bacterium strain HH^T oxidized propionate to acetate and CH_4 with either *Methanospirillum hungatei* JF-1^T or *Methanobacterium formicicum* MF^{NT}, but not with *Methanobrevibacter arboriphilus* AZ, which does not utilize formate as a substrate. The organism did not grow at higher temperatures (45–65 °C) with

Table 1. Bacterial oligonucleotide probe Eub338 and specific probes directed against the type A and type B 16S rRNA sequences of strain HH^T

Probe	Label	Sequence (5′–3′)
Eub338	Fluorescein	GCTGCCTCCCGTAGGAGT
SPA177	Fluorescein	TGTTCCCTTGAGCGTATC
SPB177	Cy3	GTTTTCCGTGAGCGTATC

Methanothermobacter thermautotrophicus strains ΔH^T and Z245. The syntrophic culture converted 1 mol propionate to about 0·9 mol acetate and 0·7 mol CH_4. The growth rate of strain HH^T depended strongly on the number of methanogens present. During enrichment, only 10 % (v/v) of a H_2/CO_2-grown culture of *Methanospirillum hungatei* JF-1^T was added, and the maximum doubling time observed in these cultures was only approximately 7 days. For further isolation procedures, the culture was inoculated into batches in which *Methanospirillum hungatei* JF-1^T was pregrown on H_2/CO_2. In these batches, the doubling time was close to 3 days, most likely as a result of the high density of *Methanospirillum hungatei* JF-1^T. The time needed for a 10^9-diluted culture to show visible growth suggests that the doubling time is even shorter under optimal conditions, approximately 1·5 days. Yeast extract (1 g l^{-1}) and fumarate stimulated growth, but the culture was not able to grow on these compounds in the absence of propionate and *Methanospirillum hungatei* JF-1^T. When fumarate (20 mM) was added, propionate was degraded more rapidly and, in addition to acetate, malate and succinate were produced. Addition of fumarate did not affect the biomass yield, which was determined by measuring the optical density. Other substrates which were tested for syntrophic growth (at 20 mM unless indicated), but which were not utilized, included lactate, pyruvate, fumarate, malate, succinate, acetate, citrate, α-ketoglutarate, butyrate, 3-hydroxybutyrate, 4-hydroxybutyrate, isobutyrate, crotonate, benzoate, glucose, fructose, xylose, methanol, ethanol, isopropanol, propanol, glycerol, butanediol, glycine, aspartate, serine, alanine, glutamate and proline (5 mM). The electron acceptors sulfate, thiosulfate, sulfite, nitrate, chlorate, iron(III) EDTA, fumarate, proline, glycine, crotonate, anthraquinone disulfonate (2·5 mM) and elemental sulfur (4 g l^{-1}) did not support axenic growth of pasteurized cultures in the presence of propionate. Pasteurized cultures did not grow on H_2/CO_2, formate or pyruvate.

Most syntrophic propionate-oxidizing bacteria use the randomizing methylmalonyl CoA pathway to oxidize propionate (Houwen *et al.*, 1990, 1991; Plugge *et al.*, 1993). An alternative pathway, which has recently been proposed for *Smithella propionica* LYP^T, explains the presence of a non-randomizing pathway in methanogenic ecosystems and enrichment cultures (de Bok *et al.*, 2001). A ^{13}C-NMR experiment with strain HH^T grown on 3-^{13}C-propionate yielded both 1- and 2-^{13}C-acetate, indicating that the organism uses the methylmalonyl CoA pathway (data not shown). The presence of this pathway has also been demonstrated in the closely related *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* TPO^T (Plugge *et al.*, 2002) by measuring some of the key enzymes of the pathway. We tested strain HH^T for axenic growth on several intermediates of this pathway, including several combinations with propionate, but growth was not observed in any of these cultures. As indicated above, fumarate stimulated syntrophic growth on propionate in this organism, probably by preventing the energetically unfavourable oxidation of succinate to fumarate during propionate oxidation. *Syntrophobacter* species are able to ferment intermediates due to their ability to couple the energetically unfavourable oxidations to fumarate reduction. Apparently, strain HH^T is not able to couple the electrons released in the three oxidation steps during propionate oxidation to fumarate reduction. Thus, strain HH^T seems to represent the first true obligately syntrophic anaerobic bacterium.

Comparative sequence analysis of the 16S rRNA gene sequences derived from the enrichment culture (spore A and spore B) with other sequences revealed that strain HH^T clusters phylogenetically with species of the genus *Desulfotomaculum* and is most closely related to *Pelotomaculum* strain FP, an organism for which two sequences (*rrnA* and *rrnB*) have also been deposited in GenBank (accession numbers AB159557 and AB159558; similarity values of 98·6 % for the type A sequence and the *rrnA* sequence and 99·8 % for the type B sequence and the *rrnB* sequence). This organism is also a mesophilic, spore-forming, syntrophic propionate-oxidizing bacterium (H. Imachi, personal communication). The closest relative which has been studied in more detail is *Cryptanaerobacter phenolicus* LR7.2^T (95·6 and 96·7 % sequence similarity to the type A and type B sequences, respectively), an anaerobic bacterium that transforms phenol and 4-hydroxybenzoate into benzoate (Juteau *et al.*, 2005). This organism was not tested for syntrophic growth on propionate or other compounds. Another related bacterium, the thermophilic propionate-oxidizer *Pelotomaculum thermopropionicum* SI^T, does grow syntrophically on propionate and is also unable to reduce sulfate (Imachi *et al.*, 2002). Within the genus *Desulfotomaculum*, another thermophilic, spore-forming, syntrophic propionate-oxidizing bacterium (strain TPO^T) was also described recently (Plugge *et al.*, 2002). Unlike these two thermophilic strains, we were not able to grow strain HH^T axenically, despite testing all substrates that supported growth of other syntrophs. Wu *et al.* (1992) described a mesophilic, spore-forming, propionate-oxidizing bacterium. This strain (strain PT) was isolated in co-culture with *Methanobacterium formicicum* MF^{NT} and was one of the prevalent syntrophic propionate oxidizers in the UASB granules they studied. Although somewhat shorter in length, cells of strain PT had the same characteristics as the bacterium described here; the strain produced endospores and was not able to reduce sulfate in the presence of propionate (Wu *et al.*, 1992). Besides butyrate, strain PT was not tested for growth on other substrates and, unfortunately, no phylogenetic information is available for this organism. Using stable isotope probing, it was demonstrated that, in addition to *Syntrophobacter* spp. and *Smithella* spp., *Pelotomaculum* spp. are important in propionate oxidation in anoxic paddy soil (Lueders *et al.*, 2004).

Based on its physiological properties and 16S rRNA gene sequence similarity to *Pelotomaculum thermopropionicum* SI^T, we propose that strain HH^T represents a novel species in

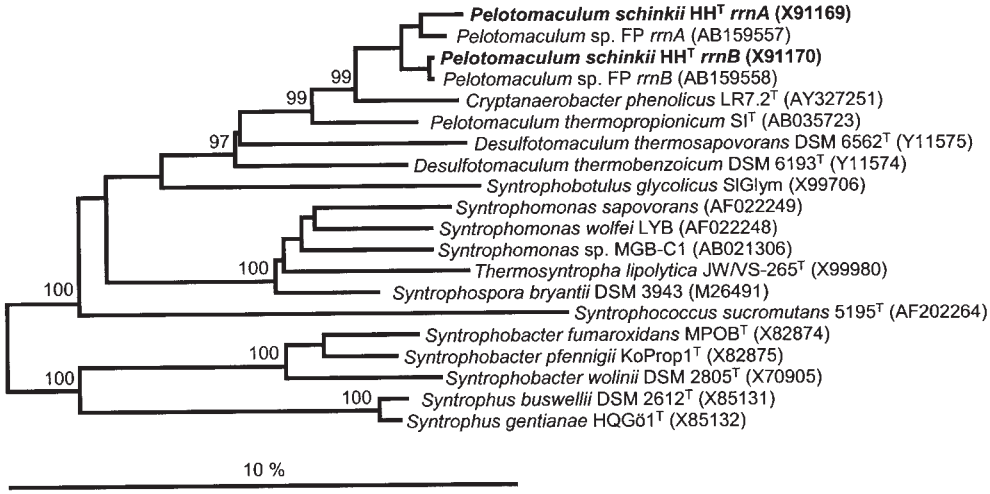

Fig. 2. Phylogenetic tree showing the relationships of the two *rrn* sequences of *Pelotomaculum schinkii* sp. nov. HH^T with published sequences. The published sequences are from the most closely related species of described bacteria and other Gram-positive and Gram-negative syntrophic bacteria. The tree is a neighbour-joining tree of a distance matrix including 1276 nucleotides between *E. coli* positions 125 and 1491. Bootstrap percentages, based on 1000 replications, at nodes display the significance of these nodes; only values above 95 % are given. Bar, 10 % sequence divergence.

the genus *Pelotomaculum*, *Pelotomaculum schinkii* sp. nov. A consensus tree based on distance-matrix and parsimony analysis is depicted in Fig. 2.

Emended description of the genus *Pelotomaculum* Imachi *et al.* 2002

Pelotomaculum (Pe.lo.to.ma′cu.lum. Gr. adj. *pelos* dark-coloured, hence anaerobic mud; L. neut. n. *tomaculum* sausage; N.L. neut. n. *Pelotomaculum* sausage-shaped bacteria living in anaerobic environments).

Unlike *Pelotomaculum thermopropionicum*, *Pelotomaculum schinkii* is mesophilic and converts only propionate in syntrophic association with hydrogenotrophic methanogens. All other characteristics of the genus as described by Imachi *et al.* (2002) remain unaffected.

Description of *Pelotomaculum schinkii* sp. nov.

Pelotomaculum schinkii (schin′ki.i. N.L. gen. n. *schinkii* named after Bernhard Schink, who studied several syntrophic conversions. The first spore-forming syntrophic bacterium, *Syntrophospora bryantii*, was isolated by his group).

Gram-positive rods, 1 × 2–2·5 μm in size, which produce spherical endospores in the late exponential phase; non-motile, strictly anaerobic. Grows syntrophically on propionate with methanogens that utilize both hydrogen and formate, such as *Methanospirillum hungatei* JF-1^T and *Methanobacterium formicicum* MF^{NT}. No organic supplements are required for growth, but yeast extract (0·1 %) and fumarate (10–20 mM) enhance growth. Habitat: anaerobic mesophilic granular sludge.

The type strain, HH^T (=ATCC BAA-615^T=DSM 15200^T), was isolated from freeze-dried granular sludge from a UASB reactor treating sugar beet waste in co-culture with *Methanospirillum hungatei* JF-1^T.

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References

Amann, R. I. (1996). In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In *Molecular Microbial Ecology Manual*, pp. 3.3.6.1–3.3.6.15. Edited by A. D. L. Akkermans, J. D. van Elsas & F. J. de Bruijn. Dordrecht: Kluwer Academic.

Bryant, M. P. (1976). The microbiology of anaerobic digestion and methanogenesis with special reference to sewage. In *Microbial Energy Conversion*, pp. 107–118. Edited by H. G. Schlegel & J. Barnes. Göttingen: E. Goltze K.G.

Church, G. M. & Gilbert, W. (1984). Genomic sequencing. *Proc Natl Acad Sci U S A* **81**, 1991–1995.

de Bok, F. A. M., Stams, A. J. M., Dijkema, C. & Boone, D. R. (2001). Pathway of propionate oxidation by a syntrophic culture of *Smithella propionica* and *Methanospirillum hungatei*. *Appl Environ Microbiol* **67**, 1800–1804.

de Bok, F. A. M., Plugge, C. M. & Stams, A. J. M. (2004). Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Res* **38**, 1368–1375.

Harmsen, H. J. M., Wullings, B., Akkermans, A. D. L., Ludwig, W. & Stams, A. J. M. (1993). Phylogenetic analysis of *Syntrophobacter*

wolinii reveals a relationship with sulfate-reducing bacteria. *Arch Microbiol* **160**, 238–240.

Harmsen, H. J. M., Kengen, K. M. P., Akkermans, A. D. L. & Stams, A. J. M. (1995). Phylogenetic analysis of two syntrophic propionate-oxidizing bacteria in enrichment cultures. *Syst Appl Microbiol* **18**, 67–73.

Harmsen, H. J. M., Kengen, H. M. P., Akkermans, A. D. L., Stams, A. J. M. & de Vos, W. M. (1996). Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by in situ hybridization using 16S rRNA-based oligonucleotide probes. *Appl Environ Microbiol* **62**, 1656–1663.

Houwen, F. P., Plokker, J., Stams, A. J. M. & Zehnder, A. J. B. (1990). Enzymatic evidence for involvement of the methylmalonyl-CoA pathway in propionate oxidation by *Syntrophobacter wolinii*. *Arch Microbiol* **155**, 52–55.

Houwen, F. P., Dijkema, C., Stams, A. J. M. & Zehnder, A. J. B. (1991). Propionate metabolism in anaerobic bacteria; determination of carboxylation reactions with ^{13}C-NMR spectroscopy. *Biochim Biophys Acta* **1056**, 126–132.

Imachi, H., Sekiguchi, Y., Kamagata, Y., Ohashi, A. & Harada, H. (2000). Cultivation and in situ detection of a thermophilic bacterium capable of oxidizing propionate in syntrophic association with hydrogenotrophic methanogens in a thermophilic methanogenic granular sludge. *Appl Environ Microbiol* **66**, 3608–3615.

Imachi, H., Sekiguchi, Y., Kamagata, Y., Hanada, S., Ohashi, A. & Harada, H. (2002). *Pelotomaculum thermopropionicum* gen. nov., sp nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium. *Int J Syst Evol Microbiol* **52**, 1729–1735.

Juteau, P., Côté, V., Duckett, M.-F., Beaudet, R., Lépine, F., Villemur, R. & Bisaillon, J.-G. (2005). *Cryptanaerobacter phenolicus* gen. nov., sp. nov., anaerobe that transforms phenol into benzoate via 4-hydroxybenzoate. *Int J Syst Evol Microbiol* **55**, 245–250.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Liu, Y. T., Balkwill, D. L., Aldrich, H. C., Drake, G. R. & Boone, D. R. (1999). Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*. *Int J Syst Bacteriol* **49**, 545–556.

Lueders, T., Pommerenke, B. & Friedrich, M. W. (2004). Stable-isotope probing of microorganisms thriving at thermodynamic limits: syntrophic propionate oxidation in flooded soil. *Appl Environ Microbiol* **70**, 5778–5786.

Nubel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W. & Backhaus, H. (1996). Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J Bacteriol* **178**, 5636–5643.

Plugge, C. M., Dijkema, C. & Stams, A. J. M. (1993). Acetyl-CoA cleavage pathway in a syntrophic propionate oxidizing bacterium growing on fumarate in the absence of methanogens. *FEMS Microbiol Lett* **110**, 71–76.

Plugge, C. M., Balk, M. & Stams, A. J. M. (2002). *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* subsp. nov., a thermophilic, syntrophic, propionate-oxidizing, spore-forming bacterium. *Int J Syst Evol Microbiol* **52**, 391–399.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463–5467.

Satokari, R. M., Vaughan, E. E., Akkermans, A. D. L., Saarela, M. & de Vos, W. M. (2001). Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **67**, 504–513.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* **61**, 262–280.

Schink, B. & Stams, A. J. M. (2002). Syntrophism among prokaryotes. In *The Prokaryotes. An Evolving Electronic Resource for the Microbiological Community*, release 3.8. Edited by M. Dworkin, K. H. Schleifer & E. Stackebrandt. New York: Springer.

Shimizu, T., Ohshima, S., Ohtani, K., Hoshino, K., Honjo, K., Hayashi, H. & Shimizu, T. (2001). Sequence heterogeneity of the ten rRNA operons in *Clostridium perfringens*. *Syst Appl Microbiol* **24**, 149–156.

Strunk, O. & Ludwig, W. (1995). ARB – a software environment for sequence data. Department of Microbiology, Technical University of Munich, Munich, Germany.

Wallrabenstein, C., Hauschild, E. & Schink, B. (1995). *Syntrophobacter pfennigii* sp. nov., new syntrophically propionate-oxidizing anaerobe growing in pure culture with propionate and sulfate. *Arch Microbiol* **164**, 346–352.

Wu, W.-M., Jain, M. K., De Macario, E. C., Thiele, J. H. & Zeikus, J. G. (1992). Microbial composition and characterization of prevalent methanogens and acetogens isolated from syntrophic methanogenic granules. *Appl Microbiol Biotechnol* **38**, 282–290.