***Syntrophobacter fumaroxidans* sp. nov., a syntrophic propionate-degrading sulfate-reducing bacterium**

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A syntrophic propionate-oxidizing bacterium, strain MPOB^T, was isolated from a culture enriched from anaerobic granular sludge. It oxidized propionate syntrophically in co-culture with the hydrogen- and formate-utilizing *Methanospirillum hungatei*, and was able to oxidize propionate and other organic compounds in pure culture with sulfate or fumarate as the electron acceptor. Additionally, it fermented fumarate. 16S rRNA sequence analysis revealed a relationship with *Syntrophobacter wolinii* and *Syntrophobacter pfennigii*. The G+C content of its DNA was 60.6 mol%, which is in the same range as that of other *Syntrophobacter* species. DNA–DNA hybridization studies showed less than 26% hybridization among the different genomes of *Syntrophobacter* species and strain MPOB^T. This justifies the assignment of strain MPOB^T to the genus *Syntrophobacter* as a new species. The name *Syntrophobacter fumaroxidans* is proposed; strain MPOB^T (= DSM 10017^T) is the type strain.

Keywords: *Syntrophobacter fumaroxidans* sp. nov., syntrophy, propionate oxidation

INTRODUCTION

For a long time, *Syntrophobacter wolinii* was the only described bacterium which could oxidize propionate syntrophically in co-culture with the hydrogen-consuming *Desulfovibrio* G11 (1). Several methanogenic syntrophic co-cultures were enriched, but obtaining defined co-cultures remained difficult. *S. wolinii* was only recently obtained in pure culture, and found to be able to grow on pyruvate or on propionate and sulfate (17). Two related syntrophic propionate-oxidizing bacteria, *Syntrophobacter pfennigii*, previously known as KOPR0P1, and strain HP1.1 were isolated with propionate and sulfate (18,19).

A mesophilic bacterium (strain MPOB^T) enriched by us on propionate was able to ferment fumarate to succinate and carbon dioxide without a syntrophic partner (12). This strain could oxidize propionate by using fumarate or sulfate as electron acceptors (12, 15). Similar to *S. wolinii*, strain MPOB^T oxidized propionate via the methylmalonyl-CoA pathway, but in contrast to *S. wolinii*, strain MPOB^T activates pro-

pionate by the use of HS–CoA transferase (11). 16S rRNA sequence analysis of *S. wolinii*, *S. pfennigii*, strain HP1.1 and strain MPOB^T revealed that these syntrophic bacteria are closely related and belong to the delta subclass of *Proteobacteria* (3, 4, 19). Remarkably, it was observed that another bacterium was related to this group: *Desulforhabdus amnigenus*, a sulfate-reducing bacterium which is not able to grow syntrophically on propionate (10).

Recently, we obtained a pure culture of strain MPOB^T. Its morphological and physiological characterization are presented here, and its taxonomic position within the genus *Syntrophobacter* is discussed.

METHODS

Bacterial strains, cultivation and isolation procedures. Strain MPOB^T originated from a propionate-degrading culture enriched from granular sludge of an upflow anaerobic sludge blanket reactor treating wastewater from a sugar refinery (12). *Syntrophobacter wolinii* (DSM 2805) and *Desulfobulbus propionicus* (DSM 2505) were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen Braunschweig, Germany). *Syntrophobacter pfennigii* was kindly provided by B. Schink (University of

The accession number for the sequence reported in this paper is X82874.

Table 1. Organic compounds tested as electron donors and carbon sources for strain MPOB^T

Substrate concentrations are given in parentheses in mM except where stated. CO₂ and NH₄⁺ were not measured but their formation was assumed from expected stoichiometries of conversion.

Substrate used:	Products
In co-culture with <i>M. hungatei</i>	
Propionate (20)	Acetate, CO ₂ and methane
In pure culture	
Propionate (20) + fumarate (20)	Acetate, CO ₂ and succinate
Fumarate (20)	Succinate and CO ₂
Malate (20)	Succinate and CO ₂
Aspartate	Succinate, NH ₄ ⁺ and CO ₂
H ₂ (80%) + fumarate (20)	Succinate
Formate (20) + fumarate (20)	Succinate and CO ₂
Pyruvate (20)	Acetate, succinate and CO ₂
In pure culture in presence of 20 mM sulfate	
Propionate (20)	Acetate and CO ₂
Formate (20)	CO ₂
Succinate (20)	CO ₂
Hydrogen (80%)	poor growth
Not utilized in presence of 20 mM sulfate	
Butyrate (10), isobutyrate (10), acetate (20), citrate (10), lactate (20), butanol (10), propanol (20), ethanol (20), methanol (10), glucose (20), fructose (10), xylose (10), glutamate (20)	—

Konstanz, Germany). *Desulforhabdus amnigenus* was provided by S. Oude Elferink (Wageningen Agricultural University, The Netherlands).

A previously described bicarbonate-buffered medium was used for isolation and cultivation (12). For isolation of strain MPOB^T the roll-tube-dilution method (5) and direct dilution series in liquid media with fumarate as carbon and energy sources were used. Purity was checked by growth in Wilkins-Chalgren anaerobe broth (Oxoid), and in media containing 1% yeast extract and 20 mM glucose, and by microscopy.

Phylogeny and DNA analysis. Phylogenetic analysis of the strain has been described previously (4, 10). The nucleotide sequence of strain MPOB^T was aligned using the Dedicated Comparative Sequence Editor version 2.60 alignment program with other 16S rRNA sequences derived from the NCBI collection and the Antwerp RRNA database. The sequence of strain HP1.1 was kindly provided by G. Zellner (Wageningen Agricultural University). Only nucleotides that could be unambiguously aligned were taken into account. A phylogenetic tree based on the neighbour-joining method was constructed from a distance matrix according to the two-parameter model of Kimura, as implemented in the TREECON for Windows software package (14).

DNA hybridizations were performed by filter hybridization according to Johnson *et al.* (6). Modifications of this method for DNA labelling and hybridization conditions have been described previously (8). The final stringent washing was done at 65 °C in 0.03 M NaCl, 0.003 M sodium citrate, with 1% SDS. The percentage of binding was determined by measuring the radioactive hybridization signals relative to that found in the homologous hybridizations. The G+C

content of the DNA was analysed at the DSMZ using standard methods (9,13,16).

Chemical analyses. All compounds except amino acids were measured by GC and HPLC as described previously (12). Amino acids were measured as described previously (7). The presence of cytochromes was analysed by recording air-oxidized versus dithionite-reduced spectra of cell-free extracts and membrane fractions. The types of quinones were analysed at the DSMZ by HPLC.

RESULTS AND DISCUSSION

Isolation and morphological characterization

The origin and enrichment of the syntrophic propionate-oxidizing co-culture from methanogenic granular sludge has been described previously (12). The *Methanospirillum* sp. was removed from the co-culture by continuous subculturing on malate and fumarate. However, this enrichment culture still contained a contaminating small coccoid bacterium, which could be enriched and isolated on media containing yeast extract. The roll-tube technique was applied to try to eliminate the contaminating bacteria. A culture containing about 10⁸ cells ml⁻¹ was diluted in anaerobic roll tubes with fumarate as the carbon source; this resulted after two months in the formation of yellowish colonies of about 2 mm diameter in the 10⁻⁴ and 10⁻⁵ dilutions. However, when these colonies were cultured in liquid medium with fumarate as substrate and 0.05% yeast extract, contaminating

Fig. 1. Phylogenetic tree based on rRNA sequence comparisons of MPOB^T with members of the delta subclass of *Proteobacteria*. The tree is based on a distance analysis using only those nucleotides that could be unambiguously aligned, and was constructed via the neighbour-joining method as implemented in TREECON for Windows (14). The scale bar represents 5% estimated sequence divergence. The accession numbers of the 16S rRNA sequences used are: *Syntrophobacter fumaroxidans*, X82874; *Syntrophobacter wolinii*, X70906; *Syntrophobacter pfennigii*, X82875; HP1.1, X94911; *Desulforhabdus amnigenus* X83274; *Thermodesulforhabdus norvegicus*, U25627; *Desulfacinum infernum*, L27426; *Desulfovibrio baarsii*, M34403; *Desulfomonile tiedjei*, M26635; *Syntrophus gentianae*, X85132; *Syntrophus buswellii*, X85131; '*Desulforhopalus vacuolatus*', L42613; *Desulfobulbus propionicus*, M34410; *Desulfobacterium autotrophicum*, M34409; *Desulfobacter postgatei*, M26633; *Pelobacter acidigallici*, X77216; *Geobacter metallireducens*, L07834; *Desulfovibrio acrylicus*, U32578; *Bilophila wadsworthia*, L35148.

bacteria were still found. Therefore, dilution series of this liquid enrichment were made, with fumarate as substrate and 0.05% yeast extract, to simultaneously purify the bacterium and check for growth of the contaminant. Growth was observed up to a dilution of 10⁻⁸. This dilution appeared to be free of the contaminating coccoid bacterium. This culture (strain MPOB^T) consisted only of the Gram-negative, non motile, rod-shaped bacterium (1.1–1.6 µm wide and 1.8–2.5 µm long) described previously (12).

Physiological characterization

Strain MPOB^T grew on fumarate between 20 and 40 °C with an optimum at 37 °C. The pH range was pH 6.0–8.0 with optimum growth around pH 7. Addition of 0.05% yeast extract was not necessary, but it stimulated growth. In co-culture with *M. hungatei*, strain MPOB^T had a growth rate on propionate of approximately 0.17 d⁻¹. In pure culture, lower growth rates were observed; 0.09 d⁻¹ during fermentative growth on fumarate and 0.024 d⁻¹ during growth on propionate and sulfate (15). Both *c*- and *b*-type cytochromes as well as the menaquinones MK-6 and MK-7 were present in fumarate-grown cells.

Table 2. Percentage of DNA–DNA hybridization of MPOB^T (1), *Syntrophobacter wolinii* (2), *Syntrophobacter pfennigii* (3), *Desulforhabdus amnigenus* (4) and *Desulfobulbus propionicus* (5)

DNA samples from strains mentioned in the left column were bound to the filter and hybridized with DNA samples from strains given in the top row.

	1	2	3	4	5
1	100	9.4	16.4	24.7	11.4
2	23.2	100	16.3	26.0	8.8
3	19.8	5.0	100	13.0	6.1
4	14.4	2.3	8.2	100	4.2
5	22.0	6.5	15.4	8.1	100

Strain MPOB^T utilized propionate syntrophically in co-culture with *M. hungatei*, and in pure culture using sulfate or fumarate as electron acceptors (Table 1). In these cases, it oxidized propionate stoichiometrically to acetate and CO₂ with concomitant production of methane, sulfide or succinate, respectively. Thiosulfate could also serve as an electron acceptor, but nitrate was not utilized. Strain MPOB^T fermented fumarate to succinate and CO₂, and reduced fumarate to succinate with hydrogen or formate as the electron donor (12). In addition, strain MPOB^T grew on some organic compounds (Table 1).

During the course of the experiments, it was observed that strain MPOB^T grew better when a batch of FeCl₂ was used in which the iron was partially oxidized, as indicated by the brownish colour. However, when the trace elements solution was added after autoclaving, this difference was not observed.

Taxonomy

The 16S rRNA sequence of strain MPOB^T was analysed, and compared with the sequences of *S. wolinii* and *S. pfennigii* (4). Using the 16S rRNA sequence data obtained previously, a new phylogenetic tree was constructed that includes the syntrophic propionate-oxidizing bacteria *S. wolinii*, *S. pfennigii*, strain HP1.1, strain MPOB^T and the sulfate-reducing bacterium *Desulforhabdus amnigenus*, and members of the delta subclass of *Proteobacteria* (3, 4, 10, 19) (Fig. 1). This tree clearly shows that the syntrophic propionate-oxidizing bacteria form one cluster with *Desulforhabdus amnigenus* and belong, according to the grouping of Devereux *et al.* (2), to group 7 of the delta subclass of *Proteobacteria*. This is a phylogenetically distinct group from the line of complete-oxidizing sulfate-reducing bacteria. To investigate this relationship at the species level, DNA–DNA hybridizations were performed. Labelled chromosomal DNA of each of the four strains, and *Desulfobulbus propionicus* as an unrelated strain, was hybridized with membrane-fixed chromosomal DNA

Table 3. Comparison of different syntrophic propionate-oxidizing bacteria

ND, Not determined.

Character	MPOB ^T	<i>S. wolinii</i>	<i>S. pfennigii</i>	HP1.1
Morphology	Lemon-shaped	Straight rod	Straight rod	Lemon-shaped
Size*				
width (µm)	1.1–1.6	0.7–0.9	1.0–1.2	1.0–1.2
length (µm)	1.8–2.5	1.5–2.3	2.2–3.0	1.4–2.4
Motility	No	No	Yes	No
Gram stain	Negative	Negative	Negative	Negative
Gas vacuoles	No	No	Yes	No
SO ₄ ²⁻ reduction	Yes	Yes	Yes	Yes
Optimal temp. (°C)	37	ND	37	40
G + C content (mol %)	60.6	ND	57.3	56.7
References	This study	1, 17	18	19

* In a syntrophic propionate-oxidizing co-culture.

of the same strains (Table 2). The homology observed between the different strains was always below 26 %, indicating that all strains belonged to different species.

Strain MPOB^T resembles the syntrophic propionate-oxidizing species *S. wolinii* and *S. pfennigii* by its ability to grow on propionate in co-culture with *M. hungatei* and in pure culture by sulfate reduction. A comparison of propionate-degrading strains is given in Table 3. *S. wolinii* can utilize fumarate and pyruvate in pure culture (17; B. L. M. van Kujik, unpublished), and *S. pfennigii* can grow in co-culture on propanol and lactate (18). However, the utilization of aspartate, succinate and formate as substrates for sulfate reduction was not observed in these two other species. The sulfate-reducing species *Desulforhabdus amnigenus* cannot oxidize propionate syntrophically and does not utilize fumarate (10). The G + C content of DNA of strain MPOB^T was 60.6 mol %, which is slightly higher than that of other *Syntrophobacter* species (18,19).

The morphological and physiological differences mentioned above, and the results obtained from the DNA–DNA hybridization, showed that the syntrophic strains do not belong to the same species. Therefore, it seems justified to describe strain MPOB^T as a new species within the genus *Syntrophobacter*. We propose the name *Syntrophobacter fumaroxidans* sp. nov.

Description of *Syntrophobacter fumaroxidans* sp. nov.

Syntrophobacter fumaroxidans (fu.mar.ox'i.dans. M. L. neut. adj. *fumaricum* pertaining to fumaric acid (fumarate); L. adj. *oxidans* oxidizing; L. adj. *fumar-oxidans*, fumarate oxidizing).

Non motile, rod- to eye-shaped cells, 1.1–1.6 by 1.8–2.5 µm in size, with round ends, single or in pairs.

Gram-negative. Endospores are not formed. The G + C content is 60.6 ± 0.2 mol % (*n* = 4). Contains *c*- and *b*-type cytochromes, and quinones MK-6 and MK-7. Strictly anaerobic and grows syntrophically on propionate in the presence of hydrogen- and formate-utilizing bacteria or methanogens (e.g. *Methanospirillum hungatei*). Ferments fumarate, malate, aspartate and pyruvate. Propionate, succinate and formate are oxidized coupled to sulfate-reduction. Besides sulfate, thiosulfate and fumarate can serve as electron acceptors, but nitrate is not reduced. Growth is optimal in freshwater medium, pH 7.0–7.6, at 37 °C. Habitat is granular sludge from an upflow anaerobic sludge bed reactor treating sugar-beet processing wastewater. Type strain: *Syntrophobacter fumaroxidans* MPOB^T (= DSM 10017^T).

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