

Syntrophomonas zehnderi sp. nov., an anaerobe that degrades long-chain fatty acids in co-culture with *Methanobacterium formicicum*

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An anaerobic, mesophilic, syntrophic fatty-acid-oxidizing bacterium, designated strain OL-4^T, was isolated as a co-culture with *Methanobacterium formicicum* DSM 1535^{NT} from an anaerobic expanded granular sludge bed reactor used to treat an oleate-based effluent. Strain OL-4^T degraded oleate, a mono-unsaturated fatty acid, and straight-chain fatty acids C_{4:0}–C_{18:0} in syntrophic association with *Methanobacterium formicicum* DSM 1535^{NT}. Even-numbered fatty acids were degraded to acetate and methane whereas odd-numbered fatty acids were degraded to acetate, propionate and methane. Branched-chain fatty acids were not degraded. The bacterium could not grow axenically with any other substrate tested and therefore is considered to be obligately syntrophic. Fumarate, sulfate, thiosulfate, sulfur and nitrate could not serve as electron acceptors for strain OL-4^T to degrade oleate or butyrate. Cells of strain OL-4^T were curved rods, formed spores and showed a variable response to Gram staining. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain OL-4^T was most closely related to the fatty-acid-oxidizing, syntrophic bacterium *Syntrophomonas* sp. TB-6 (95% similarity), *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T (94% similarity) and *Syntrophomonas erecta* DSM 16215^T (93% similarity). In addition to this moderate similarity, phenotypic and physiological characteristics, such as obligate syntrophy, spore formation and utilization of a broader substrate range, differentiated strain OL-4^T from these *Syntrophomonas* species. Therefore strain OL-4^T represents a novel species, for which the name *Syntrophomonas zehnderi* sp. nov. is proposed. The type strain is OL-4^T (= DSM 17840^T = JCM 13948^T).

Long-chain fatty acids (LCFAs) are important intermediates in the anaerobic treatment of wastewaters containing lipids and fats. Under methanogenic conditions, LCFA degradation requires the presence of a syntrophic consortium composed of acetogenic bacteria, performing fatty acid β -oxidation, and methanogenic archaea, which consume hydrogen and acetate to low concentrations (Schink, 1997). All of the 10 species and/or subspecies of syntrophic fatty-acids degraders isolated thus far belong to the families *Syntrophomonadaceae* (McInerney, 1992; Zhao *et al.*, 1993; Wu *et al.*, 2006b) and *Syntrophaceae* (Jackson *et al.*, 1999). These micro-organisms are capable of using fatty acids with

more than four carbon atoms and up to 18 carbon atoms anaerobically. LCFAs with more than 12 carbon atoms are utilized by *Syntrophomonas sapovorans* (Roy *et al.*, 1986), *Syntrophomonas wolfei* subsp. *saponavida* (Lorowitz *et al.*, 1989), *Syntrophomonas curvata* (Zhang *et al.*, 2004), *Thermosyntropha lipolytica* (Svetlitsnyi *et al.*, 1996) and *Syntrophus aciditrophicus* (Jackson *et al.*, 1999). The capability to utilize mono- and/or poly-unsaturated LCFAs (with more than 12 carbon atoms) is restricted to *Syntrophomonas sapovorans* (Roy *et al.*, 1986), *Syntrophomonas curvata* (Zhang *et al.*, 2004) and *Thermosyntropha lipolytica* (Svetlitsnyi *et al.*, 1996). The biochemical mechanisms of anaerobic degradation of unsaturated LCFAs are still poorly understood. Weng & Jeris (1976) suggested that the degradation of unsaturated LCFAs starts with chain saturation, whereas other authors report that direct β -oxidation of unsaturated LCFAs occurs (Roy *et al.*, 1986; Lalman & Bagley, 2000, 2001).

Abbreviations: BrES, 2-bromoethanesulfonic acid; DGGE, denaturing gradient gel electrophoresis; EGSB, expanded granular sludge bed; LCFA, long-chain fatty acid; VFA, volatile fatty acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain OL-4^T is DQ898277.

In this study, a novel spore-forming, syntrophic bacterium, strain OL-4^T, is described. Strain OL-4^T was obtained as a defined co-culture with *Methanobacterium formicicum* DSM 1535^{NT} and could not grow axenically on any of the substrates tested. Strain OL-4^T degraded saturated fatty acids with 4–18 carbon atoms (C_{4:0} to C_{18:0}) and the mono-unsaturated fatty-acid oleate (C_{18:1}), in co-culture with the methanogen. Phylogenetic analysis based on 16S rRNA gene sequences indicated affiliation to the genus *Syntrophomonas*. Strain OL-4^T was only moderately related to the recognized species of this genus, with ≤95% 16S rRNA gene sequence similarity, and possesses some unique physiological features. Therefore, the creation of a novel species of *Syntrophomonas* is proposed.

Strain OL-4^T was isolated in co-culture with *Methanobacterium formicicum* DSM 1535^{NT} from an oleate-degrading enrichment culture seeded with anaerobic sludge collected from a laboratory-scale, mesophilic, anaerobic expanded granular sludge bed (EGSB) reactor used to treat oleate-based effluent (University of Minho, Braga, Portugal).

A bicarbonate-buffered mineral salt medium (Stams *et al.*, 1993) was used for isolation and routine cultivation of strain OL-4^T in co-culture with *Methanobacterium formicicum* DSM 1535^{NT}. The same medium supplemented with 2% agar (Noble agar; Difco) was used to prepare roll-tubes and medium containing 0.75% agar was used for slanted soft-agar bottles. The bottles and roll-tubes were flushed with a mixture of N₂/CO₂ (80:20; 1.7 × 10⁵ Pa). *Methanobacterium formicicum* DSM 1535^{NT} and *Methanothermobacter thermautotrophicus* DSM 1053^T were kindly provided by Caroline Plugge (Laboratory of Microbiology, Wageningen University, The Netherlands) and were cultured using bicarbonate-buffered mineral salt medium and a gas phase of H₂/CO₂ (80:20; 1.25 × 10⁵ Pa). Inoculation and transfers of the cultures were performed using syringes and needles. All incubations were done at 37 °C, unless stated otherwise. Basal medium containing various carbon sources was used to determine their utilization by strain OL-4^T. The same medium was used to determine the utilization of various electron acceptors by strain OL-4^T, with oleate (1 mM) or butyrate (10 mM) as carbon sources. For the electron acceptor test, methanogenic activity was inhibited by using either pasteurization (30 min, 85 °C) or addition of 2-bromoethanesulfonic acid (BrES; 10 mM). The optimum growth temperature for strain OL-4^T in co-culture with a methanogen was determined in basal medium containing 1 mM oleate at pH 7 over the temperature range 15–55 °C. *Methanobacterium formicicum* DSM 1535^{NT} was used as the methanogenic partner for the temperature range 15–40 °C, whereas *Methanothermobacter thermautotrophicus* DSM 1053^T was used for incubations at higher temperatures. After inoculation, cultures were incubated for up to 60 days to evaluate carbon source utilization, electron acceptor utilization and optimum growth temperature. LCFA degradation by strain OL-4^T in co-culture with a methanogen could be easily observed by the clearing of LCFA in solution

and/or suspension (addition of a LCFA with more than 11 carbon atoms to the medium causes an immediate turbid/white appearance). In addition, volatile fatty acids (VFAs) present in the liquid medium and methane production were measured. Growth of strain OL-4^T in the presence of various carbon sources and electron acceptors was monitored by visual and microscopic observation. All determinations were performed in duplicate with a 10% inoculum.

Methane was detected by GC (GC-14B; Shimadzu), with a packed column (molsieve 13X 60/80 mesh, 2 m in length, 2.4 mm internal diameter; Varian) and a thermal conductivity detector. The oven temperature used was 100 °C and the injector and detector temperatures were 90 and 150 °C, respectively. Argon was used as the carrier gas at a flow rate of 30 ml min⁻¹. VFAs were analysed by using high-pressure liquid chromatography of centrifuged (10 000 g, 10 min) samples of the culture media. VFAs were measured with a Polyspher OA HY column (300 × 6.5 mm; Merck) and an RI SE-61 refractive index detector (Shodex). The mobile phase was 0.01 M H₂SO₄ at a flow rate of 0.6 ml min⁻¹. The column temperature was 60 °C.

Cultures of strain OL-4^T were observed routinely by using a phase-contrast microscope (Leica). Cells from active cultures of strain OL-4^T were stained for Gram type using 2% (w/v) crystal violet and 2% (w/v) safranin S as counterstain. Exponential-phase cells of strain OL-4^T were examined with a Leica S360 scanning microscope (Leica). Samples (10 ml) were filtered through a 0.2 µm sterile filter using a vacuum filtration system. Subsequently, cells were fixed for 12 h with a 3% glutaraldehyde solution in sodium cacodylate buffer (0.05 M, pH 7.2). After fixation, samples were dehydrated stepwise (15 min per step) with ethanol solutions of increasing concentrations (10, 25, 50, 90 and 100%, v/v). Samples were mounted on stubs and sputter-coated with gold.

Genomic DNA was extracted from co-cultures of strain OL-4^T grown on oleate using a FastDNA SPIN kit for soil (Qbiogene). Bacterial diversity and/or purity of cultures was checked by using denaturing gradient gel electrophoresis (DGGE) analysis of PCR products generated using primers U968GC-f (AACGCGAAGAACCCTTAC) and L1401-r (CGGTGTGTACAAGACCC), targeting the V6–V8 region of the bacterial 16S rRNA gene (Nübel *et al.*, 1996). The thermocycling programme used for PCR-DGGE amplification was as follows: pre-denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 40 s, elongation at 72 °C for 90 s and post-elongation at 72 °C for 5 min. The reactions were subsequently stopped by cooling to 4 °C. DGGE analysis of the amplicons was done as described by Zoetendal *et al.* (2001) by using the Dcode system (Bio-Rad) with 8% (v/v) polyacrylamide gels and a denaturing gradient of 30–60%. A 100% denaturing solution was defined as 7 M urea and 40% formamide. Electrophoresis was performed for 16 h at 85 V in 0.5 × TAE buffer at 60 °C. DGGE gels were stained with AgNO₃, as described by Sanguinetti *et al.* (1994).

The 16S rRNA gene of strain OL-4^T was amplified by PCR and sequenced directly from a genomic DNA sample at BaseClear (Leiden, The Netherlands). The quality of all sequence data was assessed automatically by using an international standard software package for superior base-calling and sequence data interpretation (PHRED). Similarity searches for the 16S rRNA gene sequence (1467 bp) derived from strain OL-4^T were performed using the NCBI BLAST search program within the GenBank database (Altschul *et al.*, 1990). Alignment of the 16S rRNA sequences was performed by using the FastAligner v1.03 tool of the ARB program package (Ludwig *et al.*, 2004). The resulting alignments were checked manually and corrected where necessary, and unambiguously aligned nucleotide positions were used for construction of a 16S rRNA gene-based phylogenetic tree, using the neighbour-joining method (Saitou & Nei, 1987). Phylogenetic placement was performed in comparison with reference sequences with Felsenstein correction and application of appropriate filters at the respective phylum level.

An oleate-degrading culture was enriched from anaerobic sludge sampled from a laboratory-scale, mesophilic, anaerobic EGSB reactor used to treat oleate-based effluent. Bottles containing 50 ml sterile anaerobic mineral medium, supplemented with 1 mM oleate, were inoculated with sludge to a final concentration of 8 % (v/v) and incubated at 37 °C. After substrate depletion, 10 % (v/v) of the culture was subsequently transferred to fresh medium containing oleate (1 mM). Immediately after each transfer, 5 % (v/v) of an H₂/CO₂-grown culture of *Methanobacterium formicicum* DSM 1535^{NT} was added to the enrichment cultures. After four successive transfers, serial dilutions of the oleate-enrichment culture (10⁻¹–10⁻¹⁰) were inoculated into bottles containing soft-agar supplemented with 1 mM oleate. Similar to what was done with the liquid cultures, 5 % (v/v) of an H₂/CO₂-grown culture of *Methanobacterium formicicum* DSM 1535^{NT} was added. After incubation for 1 month, small white colonies developed in the 10⁻¹–10⁻³ dilution soft-agar bottles. Growth was not observed at higher dilutions. Seven colonies were transferred to liquid medium containing 1 mM oleate together with 5 % (v/v) of an H₂/CO₂-grown culture of *Methanobacterium formicicum* DSM 1535^{NT}. After incubation for 3–4 weeks, the turbidity (due to the presence of oleate in solution/suspension in the medium) began to decrease and methane was detected in the headspace of four of the bottles inoculated with the colonies (OL-1, OL-3, OL-4 and OL-5). Microscopic observation of these cultures allowed the identification of four different morphotypes: thin *Methanobacterium formicicum*-like rods, fast swimming micro-organisms, very few long spiral bacteria and abundant slightly curved rods that in some cases showed a terminal endospore. DGGE analysis of DNA isolated from cultures OL-1, OL-3, OL-4 and OL-5 showed the presence of one dominant band and two to three very slight bands. All the samples had similar DGGE profiles (data not shown). From this point on, several attempts were made to purify the oleate-degrading bacteria present in these cultures. Growth in liquid media containing 1 mM oleate

was not observed at dilutions greater than 10⁻² and, therefore, the use of the dilution factor during subculture was not sufficient to eliminate all contaminants. However, after two successive subcultures with growth in the 10⁻² dilution bottle, culture OL-4 showed only one contaminant, which could be easily detected as a very low and dominant band in the DGGE profile. Cloning and sequence analysis showed that this contaminant was affiliated with *Anaerobaculum mobile* (97 % similarity), a moderate thermophilic bacterium isolated from an oleate-enrichment culture. The presence of this contaminant was still observed after the addition of antibiotics to the medium, such as nalidixic acid (up to 0.2 g l⁻¹), vancomycin (0.1 g l⁻¹) and 2-phenylethyl alcohol (2.5 ml l⁻¹). Moreover, several attempts to cultivate the cultures on solid medium (bottles with soft-agar and roll-tubes) did not lead to development of colonies. The only successful attempt for purification of oleate-degrading bacteria was by using two repeated rounds of pasteurization for 30 min at 85 °C and subculturing in the presence of *Methanobacterium formicicum* DSM 1535^{NT}. Spores did not survive pasteurization for 10 min at 100 °C. After 8 months, culture OL-4 consisted of two morphotypes, *Methanobacterium formicicum* and a rod-shaped bacterium, which formed endospores. No micro-organisms other than autofluorescent rod-shaped cells and non-fluorescent curved rods were observed. A DGGE profile of this culture showed the presence of a single band corresponding to the dominant band detected previously in cultures OL-1, OL-3, OL-4 and OL-5. The low DGGE band corresponding to the contaminant was not detected (data not shown).

The purity of strain OL-4^T in co-culture was checked periodically by incubating batches in bicarbonate-buffered mineral salt medium containing glucose (10 mM), pyruvate (20 mM), yeast extract (1 g l⁻¹) and casein tryptic peptones (0.5 g l⁻¹). Brain heart infusion broth (Difco) and 50 % strength Wilkins–Chalgren anaerobe broth (Oxoid) were also used for incubation of the culture. Growth was not observed in any of these media, indicating that, using these substrates, no contaminants were present in the culture.

Cells of strain OL-4^T were slightly curved rods with round to acute ends and 0.4–0.7 µm in width and 2.0–4.0 µm in length (Fig. 1). Slight twitching motility was observed microscopically during growth with oleate. The cells stained Gram-negative in early exponential phase and Gram-positive in a later phase. The presence of terminal endospores was also observed, conferring resistance to high temperatures. The addition of antibiotics, such as nalidixic acid, vancomycin and 2-phenylethyl alcohol, did not suppress growth of strain OL-4^T.

To determine the substrate range of strain OL-4^T in co-culture with *Methanobacterium formicicum* DSM 1535^{NT}, the following organic acids were used as the sole substrate: acetate, propionate, isobutyrate and butyrate (10 mM each); isovalerate, valerate, isocaproate, caproate, enanthate,

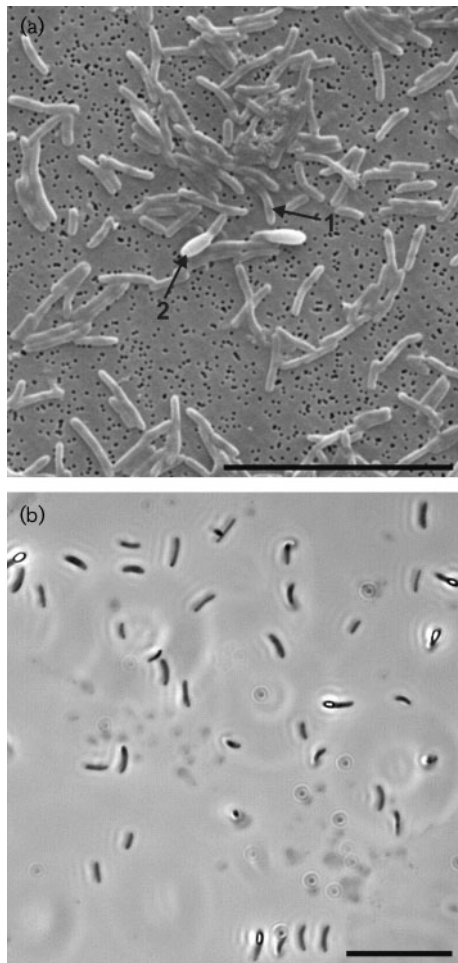


Fig. 1. Scanning electron (a) and phase-contrast (b) micrographs of cells of strain OL-4^T when grown in co-culture with *Methanobacterium formicicum* DSM 1535^{NT} on oleate (1 mM). Arrows indicate non-sporulated (1) and sporulated (2) cells. Bars, 10 µm.

caprylate and pelargonate (5 mM each); caprate, hendecanoate, laureate, myristate, valerenate, palmitate, palmitoleate, margerate, stearate, oleate and linoleate (1 mM each) (Table 1). Saturated fatty acids with an even-number of carbon atoms (C_{4:0} to C_{18:0}) were degraded by the co-culture to acetate and methane. Degradation of saturated odd-numbered (C_{5:0} to C_{17:0}) fatty acids led to the formation of propionate, in addition to acetate and methane. Oleate, a mono-unsaturated fatty acid (C_{18:1}), was used by strain OL-4^T in co-culture with a methanogen, but only very faint growth was observed with palmitoleate (C_{16:1}) and linoleate (C_{18:2}). Acetate and propionate were not used by the co-culture. Branched-chain organic acids, such as isobutyrate, isovalerate and isocaproate, were not degraded by strain OL-4^T in co-culture with *Methanobacterium formicicum* DSM 1535^{NT}. Other substrates that were tested for syntrophic growth (at 20 mM), but that were not utilized, included lactate,

pyruvate, fumarate, malate, succinate, acetate, propionate, crotonate, glucose, fructose, lactose, xylose, ribose, methanol, ethanol and glutamate. Utilization of crotonate, pyruvate and fumarate was also tested in the presence of clarified rumen fluid, but no growth was observed. Use of amino acids via the Stickland reaction was not observed in the presence of glycine (50 mM) plus alanine (25 mM) and proline (50 mM) plus alanine (25 mM). The electron acceptors sulfate, thiosulfate, sulfite, nitrate, Fe(III) EDTA, fumarate and crotonate did not support axenic growth of strain OL-4^T in the presence of oleate (1 mM) or butyrate (10 mM). Several attempts were made to grow strain OL-4^T with crotonate without the methanogenic partner. However, even after a long acclimatization stage (up to 5 months) of strain OL-4^T in co-culture with *Methanobacterium formicicum* DSM 1535^{NT} to oleate (1 mM) plus crotonate (10 mM), strain OL-4^T was not able to grow when transferred to fresh medium containing crotonate (10 mM) and BrES (10 mM). We also tested pasteurized cultures in media with crotonate, and crotonate and oleate, in the absence of BrES, but no growth occurred.

Basal medium with 1 mM oleate was used to determine the optimum temperature for growth of strain OL-4^T in co-culture. The growth temperature profile was monitored by methane production over the temperature range 15–55 °C (pH 7.0). Strain OL-4^T could grow in co-culture with a methanogen at 25–40 °C, with optimum growth occurring at 37 °C. The organism did not grow at high temperatures (45–65 °C) with *Methanothermobacter thermautotrophicus* DSM 1053^T.

Comparative sequence analysis of the 16S rRNA gene of strain OL-4^T with other sequences revealed that strain OL-4^T clustered phylogenetically with species of the genus *Syntrophomonas* and was most closely related to *Syntrophomonas* sp. TB-6 (95 % similarity). This microorganism is a mesophilic, non-spore-forming, syntrophic fatty-acid-oxidizing bacterium that degrades saturated fatty acids with four to eight carbon atoms. The closest relatives that have been studied in more detail are *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T (94 % similarity) and *Syntrophomonas erecta* DSM 16215^T (93 % similarity), syntrophic anaerobic bacteria that can degrade straight-chain fatty acids with four to eight carbon atoms in syntrophic association with methanogens (McInerney *et al.*, 1979, 1981; Beaty & McInerney, 1987; Zhang *et al.*, 2005). Unlike these two strains, strain OL-4^T could not grow axenically with any of the substrates that supported growth of other syntrophs. Furthermore, the capability to form endospores, a trait of strain OL-4^T, was not observed in members of the genus *Syntrophomonas* until the species '*Syntrophomonas erecta* subsp. *sporosyntropha*' was described recently (Wu *et al.*, 2006b). The isolation of this strain led to an amended description of the genus *Syntrophomonas*, including the possibility of spore formation by some *Syntrophomonas* strains when degrading fatty acids in co-culture with methanogens. Nevertheless, the similarity of

Table 1. Characteristics of strain OL-4^T and other mesophilic, syntrophic bacteria in the family *Syntrophomonadaceae*

Strains: 1, *Syntrophomonas bryantii* DSM 3014^T; 2, *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T; 3, *Syntrophomonas wolfei* subsp. *saponavida* DSM 4212^T; 4, *Syntrophomonas sapovorans* DSM 3441^T; 5, *Syntrophomonas curvata* DSM 15682^T; 6, '*Syntrophomonas erecta* subsp. *sporosyntrpha*' JCM 13344; 7, *Syntrophomonas* sp. strain TB-6; 8, strain OL-4^T. All strains can use saturated fatty acids with four to eight carbon atoms (C_{4:0} to C_{8:0}). Acetate (C_{2:0}) and propionate (C_{3:0}) are not degraded by any of the strains. When tested, degradation of isomers of fatty acids, such as isobutyrate (C₄), isovalerate (C₅) and isocaproate (C₆) are negative for all strains. Data are from Stieb & Schink (1985), Zhao *et al.* (1990), Wu *et al.* (2006a, b), McInerney *et al.* (1979, 1981), Beaty & McInerney (1987), Lorowitz *et al.* (1989), Roy *et al.* (1986), Zhang *et al.* (2004), Zou *et al.* (2003) and this study. ND, Not determined or not reported.

Characteristic	1	2	3	4	5	6	7	8
Cell width (µm)	0.4	0.5–1.0	0.4–0.6	0.5	0.5–0.7	0.5–0.7	0.8–1.0	0.4–0.7
Cell length (µm)	4.5–6.0	2.0–7.0	2.0–4.0	2.5	2.3–4.0	4.0–14.0	2.0–4.5	2.0–4.0
Gram reaction	Variable	–	–	–	Variable	–	–	Variable
Motility	–	+	+	+	+	+	+	+
DNA G+C content (mol%)	37.6	ND	ND	ND	46.6	40.6	47.6	ND
Growth pH (range/optimum)	6.5–7.5	ND	ND	6.3–8.1/7.3	6.3–8.4/7.5	5.5–8.4/7.0	6.0–8.9	ND
Growth temperature (range/optimum) (°C)	28–34	ND/35–37	ND	25–45/35	20–42/35–37	20–48/37–40	20–40	25–40/37
Spore formation	+	–	–	–	–	+	–	+
Substrate utilization in pure culture:*								
Crotonate	+	+	+	–	+	+	+	–
Substrate utilization in co-culture:*								
Pelargonate C _{9:0}	+	ND	+	ND	ND	ND	ND	+
Caprate C _{10:0}	+	ND	+	+	+	–	ND	+
Hendecanoate C _{11:0}	+	ND	+	+	+	ND	ND	+
Laureate C _{12:0}	–	ND	+	+	+	–	ND	+
Myristate C _{14:0}	–	ND	+	+	+	–	ND	+
Valerate C _{15:0}	–	ND	+	+	+	ND	ND	+
Palmitate C _{16:0}	–	–	+	+	+	–	–	+
Palmitoleate C _{16:1}	–	ND	ND	ND	+	ND	ND	+/-
Margarate C _{17:0}	–	ND	+	+	+	ND	ND	+
Stearate C _{18:0}	–	–	+	+	+	–	ND	+
Oleate C _{18:1}	ND	–	–	+	+	–	ND	+
Linoleate C _{18:2}	ND	ND	–	+	–	–	ND	+/-

*+, Substrate utilization; +/-, poorly utilized; –, not utilized.

strain OL-4^T to '*Syntrophomonas erecta* subsp. *sporosyntrpha*', based on 16S rRNA gene sequence, is only 93 %.

Based on its physiological properties and 16S rRNA gene sequence similarity to species of the genus *Syntrophomonas*, we propose that strain OL-4^T represents a novel species in the genus *Syntrophomonas*, with the name *Syntrophomonas zehnderi* sp. nov. A consensus tree based on distance-matrix and parsimony analysis is shown in Fig. 2.

Description of *Syntrophomonas zehnderi* sp. nov.

Syntrophomonas zehnderi (zahn'de.ri. N.L. masc. gen. n. *zehnderi* of Zehnder, named after Alexander J. B. Zehnder, who contributed to our present knowledge of the microbiology of microbial processes in methanogenic bioreactors).

Cells are slightly curved rods (approximately 0.4–0.7 µm in width and 2.0–4.0 µm in length) and show a variable

response to Gram staining. Cells have a slight twitching motility and spore formation is observed during growth on oleate in co-culture with a methanogen that utilizes hydrogen and formate. In addition to oleate, a mono-unsaturated fatty acid (C_{18:1}), straight-chain fatty acids with C_{4:0} to C_{18:0} carbon atoms can be used as sole carbon and energy sources, in co-culture with *Methanobacterium formicum* DSM 1535^{NT}. Grows at pH 7.0 and 25–40 °C in co-culture with hydrogenophilic archaea. Organic supplements are not required for growth. Even-numbered fatty acids are degraded to acetate and (presumably) hydrogen, whereas degradation of odd-numbered fatty acids also yields propionate. Acetate, propionate and branched-chain fatty acids, such as isobutyrate, isovalerate and isocaproate, do not support growth of the co-culture. Yeast extract, tryptone, peptone, glucose, lactate, pyruvate, fumarate, malate, succinate, acetate, propionate, crotonate, glucose, fructose, lactose, xylose, ribose, methanol, ethanol, glutamate, glycine plus alanine and proline plus alanine do

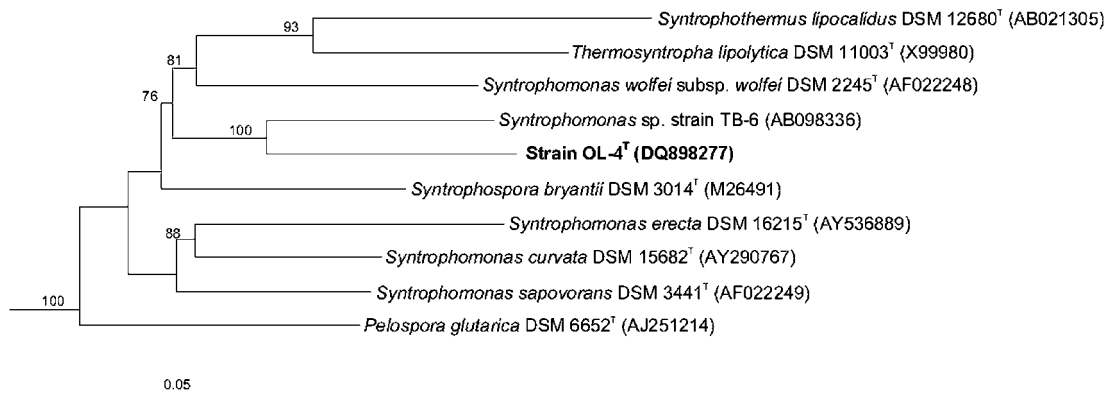


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain OL-4^T and other representatives of fatty-acid degrading bacteria. The tree was constructed using the ARB software package (Saitou & Nei, 1987), with the neighbour-joining method with Felsenstein correction. The significance of each branch is indicated at nodes by bootstrap values (%) based on 1000 replications; only values greater than 75 % are shown. GenBank accession numbers are given in parentheses. Bar, 5% sequence divergence.

not support growth. Sulfate, thiosulfate, sulfite, nitrate, Fe(III) EDTA, fumarate and crotonate do not act as electron acceptors for oleate or butyrate oxidation.

The type strain is OL-4^T (= DSM 17840^T = JCM 13948^T), which was isolated in co-culture with *Methanobacterium formicum* DSM 1535^{NT}, in Wageningen, The Netherlands, from an oleate-degrading culture enriched from anaerobic sludge sampled from an EGSB reactor used to treat oleate-based effluent (University of Minho, Braga, Portugal).

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References

- Altschul, S. F., Gish, W., Miller, W., Meyers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Beatty, P. S. & McInerney, M. J. (1987). Growth of *Syntrophomonas wolfei* in pure culture on crotonate. *Arch Microbiol* **147**, 389–393.
- Jackson, B. E., Bhupathiraju, V. K., Tanner, R. S., Woese, C. R. & McInerney, M. J. (1999). *Syntrophus aciditrophicus* sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. *Arch Microbiol* **171**, 107–114.
- Lalman, J. A. & Bagley, D. M. (2000). Anaerobic degradation and inhibitory effects of linoleic acid. *Water Res* **34**, 4220–4228.
- Lalman, J. A. & Bagley, D. M. (2001). Anaerobic degradation and methanogenic inhibitory effects of oleic and stearic acids. *Water Res* **35**, 2975–2983.
- Lorowitz, W. H., Zhao, H. & Bryant, M. P. (1989). *Syntrophomonas wolfei* subsp. *saponavida* subsp. nov., a long chain fatty-acid

degrading, anaerobic, syntrophic bacterium; *Syntrophomonas wolfei* subsp. *wolfei* subsp. nov.; and emended descriptions of the genus and species. *Int J Syst Bacteriol* **39**, 122–126.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

McInerney, M. J. (1992). The genus *Syntrophomonas*, and other syntrophic bacteria. In *The Prokaryotes*, 2nd edn, pp. 2048–2057. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. Berlin: Springer.

McInerney, M. J., Bryant, M. P. & Pfennig, N. (1979). Anaerobic bacterium that degrades fatty-acids in syntrophic association with methanogens. *Arch Microbiol* **122**, 129–135.

McInerney, M. J., Bryant, M. P., Hespell, R. B. & Costerton, J. W. (1981). *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty-acid oxidizing bacterium. *Appl Environ Microbiol* **41**, 1029–1039.

Nübel, 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.

Roy, F., Samain, E., Dubourguier, H. C. & Albagnac, G. (1986). *Syntrophomonas sapovorans* sp. nov., a new obligately proton reducing anaerobe oxidizing saturated and unsaturated long chain fatty acids. *Arch Microbiol* **145**, 142–147.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sanguinetti, C. J., Dias Neto, E. & Simpson, A. J. (1994). Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques* **17**, 914–921.

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

Stams, A. J. M., van Dijk, J. B., Dijkema, C. & Plugge, C. M. (1993). Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. *Appl Environ Microbiol* **59**, 1114–1119.

Stieb, M. & Schink, B. (1985). Anaerobic oxidation of fatty-acids by *Clostridium bryantii* sp. nov., a sporeforming, obligately syntrophic bacterium. *Arch Microbiol* **140**, 387–390.

- Svetlitshnyi, V., Rainey, F. & Wiegel, J. (1996).** *Thermosyntropha lipolytica* gen. nov., sp. nov., a lipolytic, anaerobic, alkalitolerant, thermophilic bacterium utilizing short- and long-chain fatty acids in syntrophic coculture with a methanogenic archaeum. *Int J Syst Bacteriol* **46**, 1131–1137.
- Weng, C. & Jeris, J. S. (1976).** Biochemical mechanisms in methane fermentation of glutamic and oleic acids. *Water Res* **10**, 9–18.
- Wu, C., Liu, X. & Dong, X. (2006a).** *Syntrophomonas cellicola* sp. nov., a spore-forming syntrophic bacterium isolated from a distilled-spirit-fermenting cellar and assignment of *Syntrophospora bryantii* to *Syntrophomonas bryantii* comb. nov. *Int J Syst Evol Microbiol* **56**, 2331–2335.
- Wu, C., Liu, X. & Dong, X. (2006b).** *Syntrophomonas erecta* subsp. *sporosyntropha* subsp. nov., a spore-forming bacterium that degrades short chain fatty acids in co-culture with methanogens. *Syst Appl Microbiol* **29**, 457–462.
- Zhang, C., Liu, X. & Dong, X. (2004).** *Syntrophomonas curvata* sp. nov., an anaerobe that degrades fatty acids in co-culture with methanogens. *Int J Syst Evol Microbiol* **54**, 969–973.
- Zhang, C., Liu, X. & Dong, X. (2005).** *Syntrophomonas erecta* sp. nov., a novel anaerobe that syntrophically degrades short-chain fatty acids. *Int J Syst Evol Microbiol* **55**, 799–803.
- Zhao, H., Yang, D., Woese, C. R. & Bryant, M. P. (1990).** Assignment of *Clostridium bryantii* to *Syntrophospora bryantii* gen. nov., comb. nov. on the basis of a 16S rRNA sequence analysis of its crotonate-grown pure culture. *Int J Syst Bacteriol* **40**, 40–44.
- Zhao, H., Yang, D., Woese, C. R. & Bryant, M. P. (1993).** Assignment of fatty acid- β -oxidizing syntrophic bacteria to *Syntrophomonadaceae* fam. nov. on the basis of 16S rRNA sequence analyses. *Int J Syst Bacteriol* **43**, 278–286.
- Zoetendal, E. G., Akkermans, A. D. L., Akkermans van Vliet, W. M., de Visser, J. A. G. M. & de Vos, W. M. (2001).** The host genotype affects the bacterial community in the human gastrointestinal tract. *Microb Ecol Health Dis* **13**, 129–134.
- Zou, B. Z., Takeda, K., Tonouchi, A., Akada, S. & Fujita, T. (2003).** Characteristics of an anaerobic, syntrophic, butyrate-degrading bacterium in paddy field soil. *Biosci Biotechnol Biochem* **67**, 2059–2067.