**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-4T, was isolated as a co-culture with *Methanobacterium formicicum DSM 1535NT* from an anaerobic expanded granular sludge bed reactor used to treat an oleate-based effluent. Strain OL-4T degraded oleate, a mono-unsaturated fatty acid, and straight-chain fatty acids C4:0 – C18:0 in syntrophic association with *Methanobacterium formicicum DSM 1535NT*. 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-4T to degrade oleate or butyrate. Cells of strain OL-4T 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-4T was most closely related to the fatty-acid-oxidizing, syntrophic bacterium *Syntrophomonas* sp. TB-6 (95 % similarity), *Syntrophomonas wolfei* subsp. *saponavida* DSM 2245T (94 % similarity) and *Syntrophomonas erecta* DSM 16215T (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-4T from these *Syntrophomonas* species. Therefore strain OL-4T represents a novel species, for which the name *Syntrophomonas zehnderi* sp. nov. is proposed. The type strain is OL-4T (= DSM 17840T = JCM 13948T).

Long-chain fatty acids (LCFAs) are important intermediates in the anaerobic treatment of wastewaters containing lipids and fats. Under methanogenic conditions, LCFAs are degraded by 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* (Svetlitshnyi 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* (Svetlitshnyi 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-4T 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 agar (Noble agar; Difco) was used to prepare roll-tubes. 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 H_2SO_4 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 U968G-f (AACGCGAAGAACCTTAC) and L1401-r (CGGTGTGTACAAGACC), 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 AgNO_3, 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 \(H_2/CO_2\)-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 \(H_2/CO_2\)-grown culture of *Methanobacterium formicicum* DSM 1535\(^NT\) was added. After incubation for 1 month, small white colonies developed in the 10\(^{-1}\)–10\(^{-5}\) 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 \(H_2/CO_2\)-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, enanthathe,
caprylate and pelargonate (5 mM each); caprate, hene-canoate, laureate, myristate, palmitate, palmitoleate, margerate, stearate, oleate and linoleate (1 mM each) (Table 1). Saturated fatty acids with an even-number of carbon atoms (C4:0 to C18:0) were degraded by the co-culture to acetate and methane. Degradation of saturated odd-numbered (C5:0 to C17:0) fatty acids led to the formation of propionate, in addition to acetate and methane. Oleate, a mono-unsaturated fatty acid (C18:1), was used by strain OL-4T in co-culture with a methanogen, but only very faint growth was observed with palmitoleate (C16:1) and linoleate (C18:2). 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-4T in co-culture with Methanobacterium formicicum DSM 1535NT. 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-4T in the presence of oleate (1 mM) or butyrate (10 mM). Several attempts were made to grow strain OL-4T with crotonate without the methanogenic partner. However, even after a long acclimatization stage (up to 5 months) of strain OL-4T in co-culture with Methanobacterium formicicum DSM 1535NT to oleate (1 mM) plus crotonate (10 mM), strain OL-4T 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-4T in co-culture. The growth temperature profile was monitored by methane production over the temperature range 15–55 °C (pH 7.0). Strain OL-4T 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 1053T.

Comparative sequence analysis of the 16S rRNA gene of strain OL-4T with other sequences revealed that strain OL-4T clustered phylogenetically with species of the genus Syntrophomonas and was most closely related to Syntrophomonas sp. TB-6 (95 % similarity). This micro-organism 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 2245T (94 % similarity) and Syntrophomonas erecta DSM 16215T (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-4T 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-4T, was not observed in members of the genus Syntrophomonas until the species ‘Syntrophomonas erecta subsp. sporosyntheta’ 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

Fig. 1. Scanning electron (a) and phase-contrast (b) micrographs of cells of strain OL-4T when grown in co-culture with Methanobacterium formicicum DSM 1535NT on oleate (1 mM). Arrows indicate non-sporulated (1) and sporulated (2) cells. Bars, 10 μm.
Table 1. Characteristics of strain OL-4T and other mesophilic, syntrophic bacteria in the family Syntrophomonadaceae

Strains: 1, Syntrophomonas bryantii DSM 3014T; 2, Syntrophomonas wofei subsp. wofei DSM 2245T; 3, Syntrophomonas wofei subsp. sapovida DSM 4212T; 4, Syntrophomonas sapovorans DSM 3441T; 5, Syntrophomonas curvata DSM 15682T; 6, ‘Syntrophomonas erecta subsp. sporosyntropha’ ICM 13344; 7, Syntrophomonas sp. strain TB-6; 8, strain OL-4T. All strains can use saturated fatty acids with four to eight carbon atoms (C4:0 to C8:0). Acetate (C2:0) and propionate (C3:0) are not degraded by any of the 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.

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<td>6.3–8.1/7.3</td>
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Substrate utilization in pure culture:*

- Crotonate

Substrate utilization in co-culture:*

- Pelargonate C9:0
- Caprate C10:0
- Hendecanoate C11:0
- Laureate C12:0
- Myristate C14:0
- Valerenate C15:0
- Palmitate C16:0
- Palmitoleate C16:1
- Margerate C17:0
- Stearate C18:0
- Oleate C18:1
- Linoleate C18:2

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

strain OL-4T to ‘Syntrophomonas erecta subsp. sporosyn- tropha’, 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-4T 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 (zehn’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 (C18:1), straight-chain fatty acids with C4:0 to C18:0 carbon atoms can be used as sole carbon and energy sources, in co-culture with Methanobacterium formicicum DSM 1535NT. 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
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\textsuperscript{T} (= DSM 17840\textsuperscript{T} = JCM 13948\textsuperscript{T}), which was isolated in co-culture with Methanobacterium formicicum DSM 1535\textsuperscript{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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