Syntrophomonas palmitatica sp. nov., an anaerobic, syntrophic, long-chain fatty-acid-oxidizing bacterium isolated from methanogenic sludge

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A mesophilic, syntrophic, fatty-acid-oxidizing anaerobic strain, designated MPA[T], was isolated from granular sludge in a mesophilic upflow anaerobic sludge blanket reactor used to treat palm oil mill effluent. Cells were slightly curved, non-motile rods. Spore formation was not observed. The optimal temperature for growth was around 37 °C and optimal pH for growth was 7.0. Strain MPA[T] was able to grow on crotonate or pentenoate plus butyrate in pure culture. In co-culture with the hydrogenotrophic methanogen *Methanospirillum hungatei*, strain MPA[T] was able to oxidize straight-chain saturated fatty acids with carbon chain lengths of C4–C18. The strain was unable to utilize sulfate, sulfite, thiosulfate, nitrate, fumarate, iron(III) or DMSO as an electron acceptor. The G + C content of the DNA was 45.0 mol%. Based on comparative 16S rRNA gene sequence analysis, strain MPA[T] was found to be a member of the genus *Syntrophomonas* and was most closely related to the type strains of *Syntrophomonas curvata* and *Syntrophomonas sapovorans* (sequence similarities of 94 %). Genetic and phenotypic characteristics demonstrated that strain MPA[T] represents a novel species, for which the name *Syntrophomonas palmitatica* sp. nov. is proposed. The type strain is MPA[T] (=JCM 14374[T]=NBRC 102128[T]=DSM 18709[T]).

Natural lipids such as fats and oils are hydrolysed to long-chain fatty acids (LCFAs) and glycerol. Under methanogenic conditions, LCFAs are further degraded by the syntrophic association of LCFA-oxidizing, hydrogen (and/or formate)-producing fermentative bacteria and hydrogenotrophic methanogens, because the oxidation of LCFAs is thermodynamically unfavourable in such environments unless the consumption of hydrogen and/or formate is coupled with oxidation (Schink, 1997). Therefore, LCFA-degrading anaerobes can gain only a small amount of energy through these syntrophic reactions and thus their growth is generally slow. In addition, LCFAs can cause

substrate toxicity in microbes. Consequently, isolation of LCFA-degrading bacteria has been difficult and, at the time of writing, only six species/subspecies have been described. Five of these belong to the family *Syntrophomonadaceae* within the phylum *Firmicutes*: *Syntrophomonas sapovorans* (Roy *et al.*, 1986), *Syntrophomonas wolfei* subsp. *saponavida* (Lorowitz *et al.*, 1989), *Thermosyntropha lipolytica* (Svetlitshnyi *et al.*, 1996), *Syntrophomonas curvata* (Zhang *et al.*, 2004) and the very recently described *Syntrophomonas zehnderi* (Sousa *et al.*, 2007). There is only one species described to date belonging to the class *Deltaproteobacteria*, i.e. *Syntrophus aciditrophicus* (Jackson *et al.*, 1999), which also degrades LCFAs syntrophically.

Recently, we successfully isolated strain MPA[T] from methanogenic granular sludge that was taken from palm oil mill effluent treated in a mesophilic upflow anaerobic sludge blanket reactor (Hatamoto *et al.*, 2007). In a previous study, strain MPA[T] was found to be able to grow on palmitate in co-culture with the hydrogenotrophic methanogen *Methanospirillum hungatei* JF-1[T] (DSM 864[T]).

Partial 16S rRNA gene sequence analysis revealed that strain MPA[T] was affiliated with the genus *Syntrophomonas*, but was only distantly related to any recognized species. In the present report, we describe the detailed morphological and physiological characteristics of strain MPA[T] and propose a novel species to accommodate it.

Strain MPA[T] was isolated from a methanogenic granular sludge by using conventional techniques combined with *in situ* hybridization detection via a 16S rRNA-targeted fluorescently labelled oligonucleotide probe (Hatamoto *et al.*, 2007). Media for cultivation of strain MPA[T] were prepared as described by Sekiguchi *et al.* (2000). Strain MPA[T] formed small, white–brownish lens-shaped colonies of 0.5–1 mm in diameter on agar roll tube medium with 20 mM crotonate after 1 month incubation. Cell morphologies were observed under a fluorescent microscope (Olympus BX50F). Cells of strain MPA[T] were non-motile, curved rods 1.5–4.0 µm long and 0.4–0.6 µm wide with round ends (see Supplementary Fig. S1 in IJSEM Online). Cells were Gram-negative according to the method of Hucker (Doetsch, 1981). Spores were not observed in pure culture or co-culture with *M. hungatei*.

Strain MPA[T] was strictly anaerobic as no growth occurred under trace quantities of oxygen [0.1 and 0.2 % O_2 (v/v)]. Growth in pure culture was observed on crotonate or butyrate plus pentenoate as an energy source. Yeast extract (0.05 %) was not required but did stimulate growth. For determination of the fermentation products of strain MPA[T], we used a GC-TCD (gas chromatograph equipped with thermal conductivity detector) and GC-FID (gas chromatograph equipped with flame ionization detector) as described by Kucivilize *et al.* (2003), and HPLC as described by Imachi *et al.* (2002). Fermentation products from crotonate were butyrate and acetate; 1 mol crotonate was converted to 0.2 mol butyrate and 1.5 mol acetate after 14 days (90 % electron recovery). During incubation with crotonate in pure culture, hydrogen was always below 30 Pa in the gas phase. The following substrates did not serve as sole carbon and energy source (increase of turbidity was not observed after 3 months incubation, duplicate cultures): yeast extract (0.2 %), tryptone (0.2 %), Casamino acids (0.2 %), glucose (10 mM), sucrose (10 mM), ribose (10 mM), xylose (10 mM), acetate (20 mM), propionate (10 mM), butyrate (20 mM), iso-butyrate (20 mM), straight-chain fatty acids from C5 to C8 (5 mM), straight-chain fatty acids from C10 to C18 (1 mM + 1 mM CaCl_2), oleate (1 mM) plus CaCl_2 (1 mM), linoleate (1 mM) plus CaCl_2 (1 mM), pentenoate (10 mM), fumarate (10 mM), malate (10 mM), succinate (10 mM), formate (10 mM), lactate (10 mM), glycerol (5 mM), ethanol (10 mM), 1-propanol (10 mM) and benzoate (5 mM). The following were tested as electron acceptors with butyrate (20 mM) and yeast extract (0.05 %) as electron donor, but none of them was utilized: sulfate (20 mM), sulfite (2 mM), thiosulfate (20 mM), nitrate (20 mM), fumarate (20 mM), iron(III)-nitrilotriacetate (5 mM) and DMSO (10 mM).

Substrate utilization of strain MPA[T] in co-culture with *M. hungatei* was investigated for the same substrates used in the pure culture study. Only the following substrates were used and produced methane within 3 months of cultivation: butyrate (20 mM), straight-chain saturated fatty acids from C5 to C8 (5 mM), straight-chain saturated fatty acids from C10 to C18 (1 mM + 1 mM CaCl_2) and pentenoate (10 mM). As shown in Fig. 1, palmitate was completely degraded and transformed into acetate and methane within 2 weeks of incubation in co-culture with *M. hungatei* (106 % electron recovery). The growth rate of the strain in co-culture with *M. hungatei* on palmitate (1 mM) was approximately 1.2 day^{-1} (determined by measuring methane production). Fatty acids with an even number of carbon atoms were converted into acetate and methane, whereas those with an odd number of carbon atoms were converted into propionate and methane, implying that *β*-oxidation of fatty acids was performed by the co-culture.

Effects of pH, temperature and NaCl on growth rate were determined by using the basal medium containing 10 mM crotonate plus 0.05 % yeast extract with 2 % (v/v) inoculum. Growth rate was determined by measuring the OD_{600}. To determine the optimum pH for strain MPA[T], the initial pH of the crotonate medium was adjusted to 5.5–9.0 by adding HCl or NaOH under a 100 % N_2 atmosphere at room temperature, and the strain was then incubated at 37 °C in triplicate. Under these conditions, the pH range for growth was 6.5–8.0 with optimum growth at pH 7.0. For determinations of the optimum temperature for growth, cultures were incubated at 25, 30, 35, 37, 40, 45, 50 and 55 °C in duplicate. The strain grew optimally at 37 °C; no growth was observed at 25 or 55 °C. The NaCl tolerance concentration range was 1–200 mM (growth was improved at below 25 mM NaCl) determined in duplicate incubations. Under optimum conditions (pH 7.0, 37 °C),
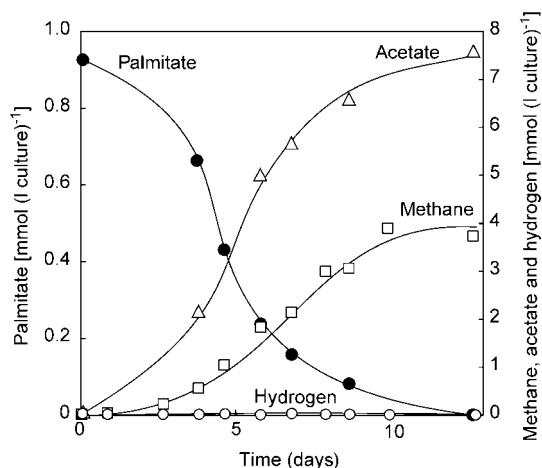

Fig. 1. Degradation of palmitate and production of acetate, methane and hydrogen by strain MPA[T] in co-culture with *M. hungatei* JF-1[T].

the growth rate of the strain in pure culture on crotonate was 1.07 ± 0.02 day^{-1} (triplicate determinations). This growth rate was about the same order as that of *Syntrophomonas wolfei* subsp. *wolfei* or the thermophilic species *Syntrophothermus lipocalidus* [0.7 and 0.9 day^{-1}, respectively (Beaty & McInerney, 1987; Sekiguchi *et al.*, 2000)] but was slower than that for *Syntrophomonas curvata* (2.2 day^{-1}; Zhang *et al.*, 2004).

The effect of antibiotics on growth of strain MPA^{T} was tested under optimum cultivation conditions by using 10 mM crotonate supplemented with 0.05 % yeast extract as a substrate. The strain was able to tolerate ampicillin (50 µg ml^{-1}), but rifampicin, chloramphenicol, kanamycin, neomycin and vancomycin (all at 50 µg ml^{-1}) completely inhibited growth.

For determination of the DNA base composition, DNA was extracted and purified according to the method of Kamagata & Mikami (1991). The DNA G+C content of strain MPA^{T} was 45.0 mol%, as determined by direct analysis of deoxyribonucleotides by HPLC (Tamaoka & Komagata, 1984). For fatty acid methyl ester (FAME) analysis, cells of strain MPA^{T} grown on 20 mM crotonate plus 0.05 % yeast extract were harvested. Extraction of cellular fatty acids and methylation were performed as described by Takai *et al.* (2004). The FAMEs were analysed via GC-MS (TRACE GC ULTRA+DSQ system; Thermo electron). The major fatty acids of strain MPA^{T} were $C_{14:0}$ (27.1 % of total fatty acids), $C_{16:0}$ (19.8 %), $C_{18:0}$ (17.5 %), $C_{16:1}$ (15.9 %), $C_{15:0}$ (7.7 %) and $C_{18:1}$ (3.8 %).

Directly extracted DNA from a pure culture of strain MPA^{T} was used for amplification of the 16S rRNA gene by using bacterial 8f and universal 1490r primer sets (Weisburg *et al.*, 1991) and sequenced by using the methods of Qiu *et al.* (2004). Sequencing results revealed that in the V1 region of the 16S rRNA gene sequence fragment peaks overlapped, suggesting that several sequences existed, but the rest of the 16S rRNA gene sequences were unambiguous. We therefore speculated that strain MPA^{T} has sequence heterogeneities in the *rrn* operons. The PCR products were purified with a MinElute PCR purification kit (Qiagen), followed by cloning with a TOPO TA cloning kit (Invitrogen). Ten clonal rRNA genes were randomly picked and sequenced. We found two types of 16S rRNA gene sequences and these were identical except for the V1 region. We designated the shorter sequence as *rrnA* (1571 bp, 6/10 clones) and the longer one as *rrnB* (1589 bp, 4/10 clones). Although we repeatedly isolated strain MPA^{T} by single colony isolation from roll tubes, there is a slight possibility that two closely related species were isolated. To exclude this possibility, fluorescence *in situ* hybridization was applied for both sequences in individual cells. Two fluorescently labelled oligonucleotide probes specific to each *rrn* sequence were designed to determine whether the *rrnA* and *rrnB* sequences belonged to a single cell. However, for a variety of hybridization conditions, we were unable to detect

positive signals from these newly designed probes (data not shown), despite observing hybridization of a previously designed strain MPA^{T}-targeted probe MPA1446 (Hatamoto *et al.*, 2007). This could be due to the relative accessibility of the target sites (Amann *et al.*, 1995). Alternatively, the 16S rRNA gene target may not be transcribed, as in *Clostridium paradoxum* (Rainey *et al.*, 1996), or the target may be transcribed but excised during rRNA maturation (Evguenieva-Hackenberg, 2005). Consequently, we confirmed the 16S rRNA gene sequence of strain MPA^{T}. We extracted total RNA, by using the method described by Sekiguchi *et al.* (2005), from crotonate-grown cultures of strain MPA^{T} and this was reverse transcribed by using SuperScript III Reverse Transcriptase (Invitrogen) with a primer complementary to the 530f primer (Lane, 1991). The resulting cDNA was used for PCR amplification with the 8f/530r primer set and the products were sequenced. Only the 16S rRNA gene sequence of *rrnA* was detected, suggesting that strain MPA^{T} has one type of 16S rRNA. Based on these results, and given the fact that multiple numbers and heterogeneity of 16S rRNA genes are common phenomena (Acinas *et al.*, 2004), we conclude that strain MPA^{T} has two types of 16S rRNA gene sequence but that only the *rrnA* type of 16S rRNA was transcribed.

Comparative 16S rRNA gene sequence phylogenetic analysis was performed as described by Hatamoto *et al.* (2007). Bootstrap resampling analysis was performed with the neighbour-joining, maximum-parsimony and maximum-likelihood methods to estimate the confidence of tree topologies, as described by Sekiguchi *et al.* (2006). A phylogenetic tree including *rrnA* and *rrnB* 16S rRNA gene sequences of strain MPA^{T} and other members of the family *Syntrophomonadaceae* was constructed (Fig. 2). The phylogenetic analysis indicated that strain MPA^{T} was affiliated with the genus *Syntrophomonas*. The closest relatives of strain MPA^{T} were the type strains of *Syntrophomonas curvata* and *Syntrophomonas sapovorans* (both having similarity values of 94 % for *rrnA* and *rrnB*).

Based on these results, strain MPA^{T} is considered to represent a member of the genus *Syntrophomonas*. Strain MPA^{T} and other *Syntrophomonas* species share common phenotypic features (Table 1), particularly syntrophic growth with hydrogenotrophic methanogens on short-chain fatty acids and the inability to utilize external electron acceptors. However, strain MPA^{T} differs from *Syntrophomonas curvata* and *Syntrophomonas sapovorans* in growth temperature and pH range for growth (Table 1). The FAME profile of strain MPA^{T} differed from those of other *Syntrophomonas* species, with strain MPA^{T} having $C_{18:0}$ as one of the predominant fatty acids (Henson *et al.*, 1988; Zhang *et al.*, 2004, 2005). Although strain MPA^{T} has almost the same substrate range as *Syntrophomonas curvata*, the mechanism of degradation of crotonate in pure culture is different, i.e. *Syntrophomonas curvata* converts 1 mol crotonate to 0.5 mol butyrate and 1 mol acetate (Zhang *et al.*, 2004). By contrast, strain MPA^{T}
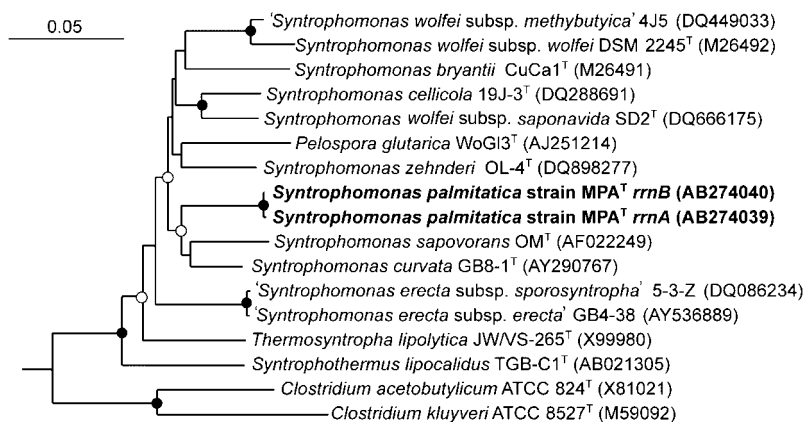
0.05

'Syntrophomonas wolfei subsp. methybutyica' 4J5 (DQ449033)
Syntrophomonas wolfei subsp. wolfei DSM 2245^T (M26492)
Syntrophomonas bryantii CuCa1^T (M26491)
Syntrophomonas cellicola 19J-3^T (DQ288691)
Syntrophomonas wolfei subsp. saponavida SD2^T (DQ666175)
Pelospora glutarica WoGl3^T (AJ251214)
Syntrophomonas zehnderi OL-4^T (DQ898277)
Syntrophomonas palmitatica strain MPA^T rrnB (AB274040)
Syntrophomonas palmitatica strain MPA^T rrnA (AB274039)
Syntrophomonas sapovorans OM^T (AF022249)
Syntrophomonas curvata GB8-1^T (AY290767)
'Syntrophomonas erecta subsp. sporosyntropha' 5-3-Z (DQ086234)
'Syntrophomonas erecta subsp. erecta' GB4-38 (AY536889)
Thermosyntropha lipolytica JW/VS-265^T (X99980)
Syntrophothermus lipocalidus TGB-C1^T (AB021305)
Clostridium acetobutylicum ATCC 824^T (X81021)
Clostridium kluyveri ATCC 8527^T (M59092)

Fig. 2. Phylogenetic position of strain MPA^T and related organisms. The tree was calculated based on a distance matrix analysis of 16S rRNA gene sequences (neighbour-joining tree). The 16S rRNA gene sequence of *Arthrobacter globiformis* DSM 20124^T (X80736) was used to root the tree (not shown). Branching points supported with probabilities above 90 % by all the analyses (based on 1000 replicates, estimated by using the neighbour-joining, maximum-parsimony and maximum-likelihood methods) are indicated by a solid circle, whereas nodes with open circles indicate >70 % bootstrap probability support by the three analyses. The accession number of each reference sequence is shown in parentheses. Bar, 0.05 nucleotide changes per sequence position.

converts 1 mol crotonate to 0.2 mol butyrate and 1.5 mol acetate, similar to *Syntrophomonas wolfei* subsp. *wolfei*. However, *Syntrophomonas wolfei* subsp. *wolfei* is unable to degrade LCFAs in co-culture with methanogens, whereas strain MPA^T can (Table 1). Based on the phenotypic features of strain MPA^T as well as its 16S rRNA gene sequence divergence, we consider that strain MPA^T represents a novel species of the genus *Syntrophomonas*,

Table 1. Characteristics of strain MPA^T (*Syntrophomonas palmitatica* sp. nov.) and related organisms in the family *Syntrophomonadaceae*

Reference strains: 1, *Syntrophomonas sapovorans* OM^T (data from Roy *et al.*, 1986; Zhang *et al.*, 2005); 2, *Syntrophomonas curvata* GB8-1^T (Zhang *et al.*, 2004, 2005); 3, *Syntrophomonas saponavida* SD2^T (Lorowitz *et al.*, 1989); 4, *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T (McInerney *et al.*, 1979, 1981; Beaty & McInerney, 1987; Zhang *et al.*, 2005); 5, *Syntrophomonas bryantii* CuCa1^T (Stieb & Schink, 1985; Zhao *et al.*, 1990; Wu *et al.*, 2006). In co-culture with methanogens, all strains can use straight-chain fatty acids with butyrate to caprylate, whereas all strains cannot use propionate. ND, Not determined; −, negative; +, positive; ±, variable.

Characteristic	Strain MPA^T	1	2	3	4	5
Cell width (µm)	0.4–0.6	0.5	0.5–0.7	0.4–0.6	0.5–1.0	0.4
Cell length (µm)	1.5–4.0	2.5	2.3–4.0	2.0–4.0	2.0–7.0	4.5–6.0
Gram reaction	−	−	±	−	−	±
Motility	−	+	+	+	+	−
DNA G+C content (mol%)	45.0	ND	46.6	ND	45.1	37.6
Growth pH (range/optimum)	6.5–8.0/7.0	6.3–8.1/7.3	6.3–8.4/7.5	ND	6.2–8.1/7.0–7.5	6.5–7.5/ND
Growth temperature (range/ optimum) (°C)	30–50/37	25–45/35–37	20–42/35–37	ND	25–45/35–37	20–40/28–34
Substrate utilization in pure culture:						
Crotonate	+	−	+	+	+	+
Butyrate+pentenoate	+	−	+	ND	−	−
Substrate utilization in co-culture with methanogens:						
Acetate	−	−	−	ND	−	ND
Caprate	+	+	+	+	−	+
Laurate	+	+	+	+	−	−
Myristate	+	+	+	+	−	−
Palmitate	+	+	+	+	−	−
Stearate	+	+	+	+	−	−
Oleate	−	+	+	−	−	ND
Linoleate	−	+	−	−	−	ND

for which the name *Syntrophomonas palmitatica* sp. nov. is proposed.

Description of *Syntrophomonas palmitatica* sp. nov.

Syntrophomonas palmitatica (pal.mi.ta′ti.ca. N.L. n. *palmitas -atis* palmitate; L. suff. *-icus -a -um* suffix used with the sense of belonging to; N.L. fem. adj. *palmitatica* belonging to palmitate).

Cells are slightly curved rods, 1.5–4.0 μm long and 0.4–0.6 μm wide. Strictly anaerobic and Gram-negative. Can grow in pure culture on crotonate or pentenoate plus butyrate. Does not utilize sulfate, sulfite, thiosulfate, nitrate, fumarate, iron(III)-nitrilotriacetate or DMSO as electron acceptors. In syntrophic association with hydrogenotrophic methanogens, can utilize straight-chain saturated fatty acids with 4–18 carbon atoms. Acetate, propionate, iso-butyrate and benzoate do not support growth of the co-culture. Temperature range for growth is 30–50 °C (optimum, 37 °C). pH range for growth is 6.5–8.0 (optimum, pH 7.0). The G+C content of the DNA of the type strain is 45.0 mol%.

The type strain, MPA^T (=JCM 14374^T=NBRC 102128^T=DSM 18709^T), was isolated from granular sludge of an upflow anaerobic sludge blanket reactor treating palm oil mill effluent.

Acknowledgements

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