Identification and Cultivation of Anaerobic, Syntrophic Long-Chain Fatty Acid-Degrading Microbes from Mesophilic and Thermophilic Methanogenic Sludges[∇]

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We investigated long-chain fatty acid (LCFA)-degrading anaerobic microbes by enrichment, isolation, and RNA-based stable isotope probing (SIP). Primary enrichment cultures were made with each of four LCFA substrates (palmitate, stearate, oleate, or linoleate, as the sole energy source) at 55°C or 37°C with two sources of anaerobic granular sludge as the inoculum. After several transfers, we obtained seven stable enrichment cultures in which LCFAs were converted to methane. The bacterial populations in these cultures were then subjected to 16S rRNA gene-based cloning, in situ hybridization, and RNA-SIP. In five of seven enrichment cultures, the predominant bacteria were affiliated with the family *Syntrophomonadaceae*. The other two enrichment cultures contained different bacterial populations in which the majority of members belonged to the phylum *Firmicutes* and the class *Deltaproteobacteria*. After several attempts to isolate these dominant bacteria, strain MPA, belonging to the family *Syntrophomonadaceae*, and strain TOL, affiliated with the phylum *Firmicutes*, were successfully isolated. Strain MPA converts palmitate to acetate and methane in syntrophic association with *Methanospirillum hungatei*. Even though strain TOL assimilated [¹³C]palmitate in the original enrichment culture, strain TOL has not shown the ability to degrade LCFAs after isolation. These results suggest that microbes involved in the degradation of LCFAs under methanogenic conditions might not belong only to the family *Syntrophomonadaceae*, as most anaerobic LCFA-degrading microbes do, but may also be found in phylogenetically diverse bacterial groups.

To date, anaerobic (methanogenic) treatment processes have been widely applied to the treatment of municipal and industrial waste and wastewater because of demonstrable performance and cost-saving advantages (30, 34, 45). To expand the applications of these processes, many engineers and researchers are now being challenged to treat more-complex waste and wastewater containing anthropogenic compounds and/or compounds that are recalcitrant to biodegradation (17). This type of treatment has been applied to lipid-rich wastes and wastewater since the early stages of development of anaerobic treatment technologies for the following reasons: (i) lipid-rich waste and wastewater are widely found in certain food processing industries such as dairy, edible oil, and slaughterhouses (22, 33); and (ii) lipids have a high theoretical methane yield in comparison with that of other organic substrates (22). However, most of the previous studies of methanogenic processes with lipid-rich wastewater found them to be less stable and able to accommodate lower organic-loading rates (see, for example, references 37 and 47) than other types of waste and wastewater. This may be due in part to the acute toxicity of long-chain fatty acids (LCFA), which are the main constituent and hydrolysate of lipids in the anaerobic consor-

tium. LCFA give rise to substrate toxicity in anaerobic microbes (10, 18, 20) and tend to adsorb onto the biomass and flow out of the reactor. In addition, it has been shown that LCFA of 16 and 18 carbons, i.e., palmitate (C₁₆), stearate (C₁₈), oleate (C_{18:1}), and linoleate (C_{18:2}), are most abundant in many types of lipid-rich wastewater (27). Therefore, efficient degradation of these LCFA is essential for the successful treatment of lipid-rich wastewater, and there is a need to better understand the microbes responsible for degradation of LCFA (in particular, C₁₆ to C₁₈) in methanogenic processes.

Under methanogenic conditions, LCFA is converted to acetate and hydrogen through β -oxidation reactions (49, 50) as well as aerobic processes. The methanogenic β -oxidation of LCFA is carried out by syntrophic LCFA-degrading, hydrogen- (and/or formate-) producing, fermentative bacteria and hydrogenotrophic methanogens, because the oxidation of LCFA is thermodynamically unfavorable in such environments unless the consumption of reducing equivalents (hydrogen and/or formate) is coupled with oxidation (39). Therefore, LCFA-degrading anaerobes can gain only a small amount of energy through syntrophic reactions, and thus, their growth is generally slow. Due to the syntrophic metabolism and toxicity of LCFA, isolation of LCFA-degrading bacteria has been difficult and only five species/subspecies have been described. The first isolate described was *Syntrophomonas sapovorans* (36) (Zhao et al. pointed out that *S. sapovorans* contains an unknown contaminating bacterium [54]), and the second isolate described was *Syntrophomonas wolfei* subsp. *saponavida* (23),

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TABLE 1. 16S rRNA-targeted oligonucleotide probes used in this study

Probe name	OPD ^a name	Target group	Probe sequence (5' to 3')	% FA ^b	Reference
EUB338	S-D-Bact-0338-a-A-18	<i>Bacteria</i>	GCTGCCTCCCGTAGGAGT	10	2
Synm700	S-F-Synm-0700-b-A-23	Mesophilic members of the family <i>Syntrophomonadaceae</i>	ACTGGTRTTCCTCCTGATTCTA	15	11
TOL1028	S-S-TOL-1028-a-A-18	Strain TOL	GCTCCCTCAGTTTCCCTC	20	This study
TSP436	S*-0436-a-A-18	Clones TPA, TST, and JA2	CCTTCGTCCCTTAGAACA	10	This study
MPA1446	S*-1446-a-A-18	Strain MPA	GGCTCTCTCTATTAAAGT	5	This study
MST445	S*-0445-a-A-18	Clone MST	CCGCATCCCTTTCTTTC	15	This study
MSP1445	S*-1445-a-A-18	Clones MOL and MLI	ACGCAGGTACCCCGAGTT	15	This study

^a OPD, oligonucleotide probe database.^b Percentage (vol/vol) of formamide (FA) in FISH buffer.

both of which are mesophiles isolated from anaerobic sludges. Subsequently, *Thermosyntropho lipolytica* (46) was isolated from an alkaline hot spring; this strain is the first thermophilic anaerobic LCFA-degrading bacterium described. *Syntrophus aciditrophicus* (15) was isolated and described as an anaerobic benzoate oxidizer that could also degrade LCFA. Recently, *Syntrophomonas curvata* (52) was isolated from mesophilic anaerobic sludges. With regard to thermophilic LCFA-degrading bacteria other than *T. lipolytica*, only two methanogenic LCFA-mineralizing enrichment cultures have also been reported (4, 32).

In this paper, we describe thermophilic and mesophilic LCFA-degrading enrichment cultures from anaerobic granular sludges for treating wastewater with high lipid concentrations. Since LCFA of 16 and 18 carbons are most abundant in the wastewater, we used palmitate, stearate, oleate, and linoleate as substrates. In our attempt to isolate LCFA-degrading microorganisms, we obtained a mesophilic palmitate-degrading isolate. An RNA-based stable isotope-probing (SIP) approach indicates that novel LCFA-degrading bacteria are present in some enrichment cultures.

MATERIALS AND METHODS

Sources of methanogenic granular sludge. Anaerobic granular sludges were taken from two laboratory-scale multistaged upflow anaerobic sludge blanket (UASB) reactors that were operated in parallel at two different temperatures of mesophilic (35°C) or thermophilic (55°C) conditions (19). The UASB reactors had been used to treat palm oil mill effluent that contained high chemical oxygen demand (COD) attributable to a high concentration of lipids (ca. 25,000 mg COD · l⁻¹). Most of the lipid COD was due to oleate (33% in total COD), palmitate (31%), linoleate (9%), and stearate (1%), with the remainder likely due to other LCFA and glycerol. Both reactors successfully treated the wastewater, with a lipid removal rate of over 80%, under high lipid-loading rates (9.9 ± 0.7 kg COD · m⁻³ · d⁻¹ and 4.1 ± 0.3 kg COD · m⁻³ · d⁻¹ for thermophilic and mesophilic reactors, respectively) (19).

Microorganisms and cultivation media. The LCFA-degrading anaerobe strain MPA was isolated in this study. Strain TOL was obtained from a thermophilic oleate-degrading enrichment culture established in this study. *Methanothermobacter thermautotrophicus* strain type II was isolated in our laboratory, and the anaerobic syntrophic ethanol-degrading bacterium *Tepidanaerobacter syntrophicus* strains JE, JL, and OL were isolated as described in our previous study (40). *Syntrophothermus lipocalidus* strain TGB-C1 (DSM 12680) was kindly provided by Yuji Sekiguchi (National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan). *Methanospirillum hungatei* strain JF1 (DSM 864), *Methanosaeta thermophila* strain PT (DSM 6194), *Syntrophomonas sapovorans* strain OM (DSM 3441), *Syntrophomonas wolfei* subsp. *saponavida* strain SD2 (DSM 4212), *Syntrophospora bryantii* strain CuCa1 (DSM 3014), *Thermosyntropho lipolytica* strain JW/VS-256 (DSM 11003), *Syntrophobacter fumaroxidans* strain MPOB (DSM 10017), *Desulfovibrio vulgaris* subsp. *vulgaris* (DSM 2119), and *Thermodesulfovibrio yellowstonii* strain YP87 (DSM 11347) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braun-

schweig, Germany). *Clostridium acetobutylicum* (JCM1419) was purchased from the Japan Collection of Microorganisms (JCM, Wako, Japan). *Escherichia coli* strain TOPO10 was purchased from Invitrogen Corp. The culture media and cultivation conditions used for enrichments and isolations of LCFA-oxidizing anaerobes were prepared as described previously (13). The four substrates used for enrichment of LCFA-oxidizing anaerobes were palmitate (1 mM), stearate (1 mM), oleate (1 mM), and linoleate (1 mM), as the sole energy sources. To reduce LCFA inhibition, 1 mM of calcium chloride was also added to the basal medium. In syntrophic growth/substrate utilization tests, *M. thermautotrophicus* strain type II (for thermophilic cultivation) or *M. hungatei* (for mesophilic cultivation) was added to the medium (10% inoculum), and growth and substrate utilization were checked by methane production. The purity of strains MPA and TOL was routinely examined by microscopy and by inoculating samples into thioglycolate medium (Difco), AC medium (Difco), and a mixed carbohydrate medium containing 0.1% yeast extract, 10 mM glucose, 10 mM sucrose, and 10 mM pyruvate at 37 and 55°C.

Construction of 16S rRNA gene clone libraries from LCFA-degrading enrichment cultures. DNA extraction from enrichment cultures was performed as described previously (51). For the construction of 16S rRNA gene clone libraries, we used the following primer set for the PCR amplification of bacterial 16S rRNA genes: EUB338F (a mixture of complementary sequences of the EUB338, EUB338-I, EUB338-II, and EUB338-III probes) (2, 6) and prokaryote-specific primer 1490R (5'-GGTACCTTGTTACGACTT-3', *E. coli* nucleotide positions 1491 to 1509) slightly modified from a report by Weisburg et al. (48). The PCR products were purified with a GENECLEAN II kit (Qbiogene), followed by cloning using a TOPO TA cloning kit (Invitrogen). Ten clonal rRNA genes were randomly picked from each clone library and subjected to restriction fragment length polymorphism (RFLP) analysis with HaeIII restriction endonuclease. Representative clones having different RFLP patterns were then subjected to sequencing.

Sequencing and phylogenetic analysis. Sequences of representative rRNA gene clones as well as the 16S rRNA gene from pure cultures were obtained as described previously (34). Sequence data were aligned with an ARB program package (24), and the aligned data were manually corrected based on information about primary and secondary structures. The phylogenetic trees based on 16S rRNA gene sequences were constructed by the neighbor-joining method (38) implemented with the ARB program. Bootstrap resampling analysis (8) for 1,000 replicates was performed to estimate the confidence of tree topologies.

Fluorescence in situ hybridization (FISH). Fixation of cells in the enrichment cultures and pure cultures and hybridizations were carried out based on a method described elsewhere (42). The 16S rRNA-targeted oligonucleotide probes used in this study are shown in Table 1. New probes were designed using ARB (24), and their specificities were confirmed by using BLAST (1) and the probe match program of the Ribosomal Database Project (28). To evaluate the specificity of the newly designed probes, the following species were used as reference strains: *S. sapovorans*, *S. wolfei* subsp. *saponavida*, *T. lipolytica*, and *S. lipocalidus*, belonging to the family *Syntrophomonadaceae* (54) for probe TSP436; *S. fumaroxidans* and *D. vulgaris* subsp. *vulgaris* for probe MST445; *T. syntrophicus* strains JE, JL, and OL and *C. acetobutylicum* for probe TOL1028; *S. sapovorans*, *S. wolfei* subsp. *saponavida*, *T. lipolytica*, and *S. lipocalidus* for probe MPA1446; and *S. bryantii*, *S. sapovorans*, and *S. wolfei* subsp. *saponavida* for probe MSP1445. Hybridization stringency was adjusted by adding formamide to the hybridization buffer (see Table 1). For double staining of the enrichment cultures, Cy-5- or Alexa Fluor 488- and Cy-3-labeled probes were simultaneously used.

TABLE 2. Stoichiometry of substrate conversion by anaerobic LCFA-degrading enrichment cultures

Enrichment culture	Substrate	Substrate converted (mmol)	Substrate production (mmol)				Electron recovery (%)
			Methane	Acetate	Butyrate	H ₂	
Mesophilic culture							
MPA	Palmitate	0.033	0.333	ND ^b	ND	ND	88
MST	Stearate	0.018	0.213	0.001	ND	ND	94
MOL	Oleate	0.032	0.182	0.256	ND	ND	105
MLI	Linoleate	0.032	0.169	0.255	ND	ND	106
Thermophilic culture							
TPA	Palmitate	0.033	0.32	ND	ND	ND	85
TST	Stearate	0.031	0.389	0.001	ND	ND	97
TOL	Oleate	0.014	0.06	0.103	0.003	ND	96
TLI	Linoleate	— ^a	—	—	—	—	—

^a —, concentrations of substrates and products were not measured because the TLI enrichment could not be transferred further.

^b ND, not detected (below detection limit).

Stable isotope probing of RNA. After flushing with N₂-CO₂ (80:20 [vol/vol]) to remove methane gas from the headspace of a pregrown enrichment culture, ¹³C-labeled palmitate ([1,2,3,4-¹³C₄]palmitic acid potassium salt; Isotech, Miamisburg, OH) was added to a concentration of 1 mM and incubated anaerobically at 37°C or 55°C. Calcium chloride (1 mM) was also added to the basal medium to reduce LCFA inhibition. When the palmitate was mostly converted to methane, the culture medium (~10 ml) was sampled and microorganisms were collected by centrifugation. Total RNA extraction from the collected microorganisms and RNA purification were conducted by a method described previously (43). Equilibrium density gradient centrifugation was performed based on methods reported by Manefield et al. (29) and Lueders et al. (26), with the following modification of the centrifugation conditions. Density gradient centrifugation was performed with 5-ml polyallomer Quick-Seal tubes in a model NVT65.2 rotor (both Beckman Coulter) at 41,500 rpm and 20°C for 48 h. RNA was precipitated by isopropanol from fractionated gradients, and rRNA was quantified by real-time reverse transcription (RT)-PCR performed with a Light-Cycler (Roche) by using a QuantiTect SYBR green RT-PCR kit (QIAGEN). A reaction mixture for RT-PCR was prepared according to the manufacturer's instructions, with the following 16S rRNA gene-targeted primer sets: EUB338F and 907r (5'-CCCCGTCATTCMTTGTGAGTTT-3'; a slightly modified version of a primer designed by Amman et al. [3]) for the domain *Bacteria*; and Ar109f (25) and Ar912r (5'-CCCCGCCAATTCCTTTAA-3'; slightly modified from a sequence reported by Lueders and Friedrich [25]) for the domain *Archaea*. A RiboMAX T7 Express system (Promega) was used to in vitro transcribe 16S rRNA from *E. coli* and *M. thermoautotrophicus* 16S rRNA gene PCR products (generated with the bacterial primer pair [T7-] 8F [47]/1490R or the archaeal primer pair [T7-] Arch21F [7]/1490R, with T7 promoter sequences attached via the forward primer). These transcription products were quantified spectrofluorometrically with a RiboGreen RNA Quantification kit (Molecular Probes) and used as standard templates. The quantitative RT-PCR conditions were as follows: reverse transcription at 50°C for 30 min and initial activation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 52°C for 20 s; extension at 72°C for 30 s and 35 s for primer pairs EUB338F/907r and Ar109f/Ar912r, respectively; and 5 s for data acquisition at 78°C or 81°C for primer pairs EUB338F/907r and Ar109f/Ar912r, respectively. After each run, a melting curve was recorded between 62°C and 95°C to confirm the specificity of the real-time PCR assays. Bacterial terminal RFLP (T-RFLP) fingerprinting from density-resolved gradient fractions was done with primers EUB338F/907r (Beckman-dye D4), and PCR products were digested by MspI restriction endonuclease. The digested sample and a DNA size standard kit 600 (Beckman Coulter) were prepared according to the manufacturer's instructions. Electrophoresis was performed on a CEQ-2000XL apparatus (Beckman Coulter) equipped with a CEQ separation capillary array 33-75B under the following conditions: denaturation at 90°C for 120 s, 30-s injection time, 2.0-kV injection voltage, and 6.0-kV run voltage at 50°C for 60 min. RNA from selected fractions was reverse transcribed with 1490R (48) for domains *Bacteria* and *Archaea*, and bacterial 16S rRNA gene clone libraries were constructed as described above.

Microscopy and analytical methods. An Olympus microscope equipped for epifluorescence was used for studies of cell morphology and epifluorescence (Olympus BX50F). LCFA was extracted with 10 ml hexane-isopropanol (5:3 [vol/vol]). After mixing, the hexane phase was transferred to a glass tube, and hexane was evaporated by heating the tube in a 60°C water bath under a nitrogen

atmosphere. LCFA was measured by gas chromatography (Shimadzu-GC14-B with flame ionization detector; packing material, Advance-DS; column temperature, 180°C for 6 min, then increasing at a rate of 10°C/min, ending at 220°C; carrier gas, nitrogen). Concentrations of short-chain fatty acids, methane, hydrogen, and carbon dioxide were determined by gas chromatography (19).

Nucleotide sequence accession numbers. The 16S rRNA gene sequence data obtained in this study were deposited in the GenBank/EMBL/DBJ databases under accession numbers AB232551 to AB232580.

RESULTS

Enrichment of LCFA-degrading microbes under mesophilic and thermophilic conditions. We started the experiment with eight enrichment cultures (two types of sludges and four substrates) initially. Eight enrichment cultures were made as follows: an enrichment with palmitate from mesophilic granular sludge (MPA); an enrichment with stearate from mesophilic granular sludge (MST); an enrichment with oleate from mesophilic granular sludge (MOL); an enrichment with linoleate from mesophilic granular sludge (MLI); an enrichment with palmitate from thermophilic granular sludge (TPA); an enrichment with stearate from thermophilic granular sludge (TST); an enrichment with oleate from thermophilic granular sludge (TOL); and an enrichment with linoleate from thermophilic granular sludge (TLI). Growth and methane production were observed in all eight primary enrichment cultures after 2 to 3 months of incubation. These cultures were successively transferred into fresh medium with 5 to 10% (vol/vol) inoculation. In some cases, the enrichment cultures exhibited unstable conditions such as unexpected lag time for growth and stagnation of growth. As the enrichment progressed, mesophilic cultures MPA and MOL showed measurable growth and reduction of LCFA after about 1 month, while MST and MLI always required 2 to 3 months for complete mineralization of substrate LCFA. In the thermophilic enrichment cultures, TPA, TST, and TOL could grow within 2 weeks after several transfers; however, enrichment culture TLI could not grow after three successive transfers even when a large inoculum, 25% (vol/vol), was used. Following the loss of enrichment culture TLI, we used the seven remaining cultures for further analysis.

Microscopic observation of the three thermophilic and four mesophilic enrichment cultures revealed that all contained rod-shaped F₄₂₀ autofluorescent methanogenic cells, *Methanosaeta*-like thick rods, and at least two to four morphologically distinct microorganisms (data not shown). All enrichments de-

FIG. 1. Phylogenetic affiliations of representative clones from each enrichment culture based on comparative analyses of 16S rRNA gene sequences. (A) The clones TPA and TST (retrieved from thermophilic palmitate and stearate enrichments) and clones MLI and MOL (obtained from enrichments of mesophilic linoleate and oleate) belong to the family *Syntrophomonadaceae*; (B) the clone TOL, from thermophilic oleate enrichment, belongs to the phylum *Firmicutes*; (C) the clone MST, derived from mesophilic stearate enrichment, is affiliated with the class *Deltaproteobacteria*. The scale bars represent the number of nucleotide changes per sequence position. The symbols at the nodes indicate the bootstrap values obtained with 1,000 resampling analyses.

graded LCFA and produced methane (Table 2). We therefore considered that in our enrichment cultures, syntrophic LCFA-oxidizing bacteria and hydrogenotrophic methanogens carried out LCFA degradation to methane and that acetate was further oxidized by aceticlastic methanogens. The specific growth rates of the enrichment cultures based on methane production were approximately 0.50 day^{-1} , 0.48 day^{-1} , and 0.80 day^{-1} for thermophilic enrichment cultures TPA, TST, and TOL, respectively, and 0.74 day^{-1} , 0.29 day^{-1} , 0.41 day^{-1} , and 0.06 day^{-1} for mesophilic enrichment cultures MPA, MOL, MST, and MLI, respectively.

Phylogenetic analysis of bacterial populations in LCFA-degrading enrichment cultures. To identify the bacteria contributing to the degradation of LCFA, we first constructed bacte-

rial 16S rRNA gene clone libraries using the DNA that was extracted from the enrichment cultures over five successive transfers. We obtained two to five types of RFLP patterns from each of these enrichment cultures. The clone libraries from thermophilic enrichment cultures TPA and TST were dominated by bacteria affiliated with the family *Syntrophomonadaceae*. The most closely related member of that family is *S. lipocalidus*, but 16S rRNA gene sequence similarity is only about 91% (Fig. 1A). In contrast, the predominant clone recovered from enrichment culture TOL was related to a deeply branched lineage of the phylum *Firmicutes*, not closely related to any other microbes except for our recent isolate, *T. syntrophicus* (sequence similarity 91%) (Fig. 1B). In the clone libraries of mesophilic enrichment cultures MPA, MOL, and MLI,

FIG. 3. Cesium trifluoroacetate density gradient centrifugation of RNA extracted from enrichment cultures grown with $[1,2,3,4-^{13}\text{C}_4]$ palmitic acid potassium salt (A) enrichment culture TPA, (B) enrichment culture TOL, and (C) enrichment culture MST. The abundance of bacterial rRNA was quantified with real-time reverse transcription-PCR. Fractions indicated by arrows were used for cloning analysis.

clones affiliated with the genus *Syntrophomonas* of the family *Syntrophomonadaceae* were most common, with *S. curvata* and *S. sapovorans* as the closest cultivated relatives (sequence similarities, 94 to 95%) (Fig. 1A). In the enrichment culture MST, clones belonging to the class *Deltaproteobacteria* were most abundant, but there were no closely related cultivated species. The most closely related species were some *Desulfovibrio* spp. and *Desulfomicrobium* spp. with around 85% sequence similarities (Fig. 1C).

Next, we made use of five specific oligonucleotide probes designed in this study (Table 1) to confirm whether the recovered clones actually represented the predominant bacterial populations in the enrichment cultures. For the evaluation of probe specificity, the newly designed probes TSP436, TOL1028, MPA1446, MST445, and MSP1445 were first applied to FISH analysis with reference organisms (see Materials and Methods). Although all reference bacteria had positive signals with probe EUB338, no signals were detected with the newly designed probes at any formamide concentration in hybridization and washing buffers (data not shown). Short, curved rod-shaped cells reacted with probes MPA1446 and MSP1445 in the mesophilic enrichment cultures MPA, MOL, and MLI (Fig. 2A, C, and D), while in the mesophilic stearate enrichment culture MST, a rod-shaped bacterium gave a positive signal with probe MST445 (Fig. 2B). On the other hand, in thermophilic enrichment cultures TPA and TST, probe TSP436 hybridized with a slightly curved, short rod-shaped bacterium (Fig. 2E and F). In the TOL enrichment culture, a straight or slightly curved rod-shaped bacterium reacted with probe TOL1028 (Fig. 2G). Each probe specifically detected cells in an enrichment-specific manner; e.g., probe TSP436 did not detect positive cells in enrichments TOL, MPA, MST,

MOL, and MLI. These findings indicate that the probes designed in this study were sufficiently specific for each clone and that each enrichment culture consisted mainly of one particular type of bacteria. In addition, these five probes could detect most of the EUB338-positive cells in each enrichment (Fig. 2), and these positive cells were always dominant after this time.

Estimation of LCFA-degrading microbes in enrichment culture by RNA-SIP. The enrichment cultures TOL and MST had dominant microbes with no known relatives capable of degrading LCFA syntrophically. To elucidate whether the dominant microbes in enrichment cultures TOL and MST might be carrying out the observed LCFA degradation, we applied RNA-based SIP analysis to these cultures and also to TPA for a positive reference. We supplemented ^{13}C -labeled palmitate ($[1,2,3,4-^{13}\text{C}_4]$ palmitic acid potassium salt) at a concentration of 1 mM, which is the same concentration as in the enrichment culture. This palmitate has four carbon atoms on the carboxyl end labeled with ^{13}C . As LCFA degradation is carried out via β -oxidation, which proceeds by the removal of two-carbon units from the carboxyl end of LCFA, these ^{13}C -labeled carbons could be mineralized only by LCFA degraders. After ^{13}C -labeled palmitate was consumed, the RNA profile was shifted toward a heavier cesium trifluoroacetate-buoyant density (Fig. 3). Bacterial communities resolved within the centrifugation gradients were analyzed by T-RFLP fingerprinting and cloning analysis. In the heavy-gradient portion of RNA from enrichment culture TOL, a 67-bp T-RF clone, which has the same sequence as that of clone TOL, was dominant, representing 9 out of 10 retrieved clones. In the heavy-gradient portion of RNA from enrichment culture MST, a 247-bp T-RF clone, which has the same sequence as that of clone MST, was dominant, representing 7 out of 10 clones retrieved. These results

FIG. 2. In situ hybridization of LCFA-degrading enrichment cultures. The enrichment cultures were simultaneously hybridized with Cy-5- or Alexa-488-labeled *Bacteria* domain-specific probe EUB338 (green, panel 3) and Cy-3-labeled clone-specific probes (red, panel 2). Phase-contrast micrographs (panel 1) and fluorescence micrographs of the same fields (panels 2 and 3) are shown. (A) Mesophilic palmitate culture (enrichment MPA) hybridized with probe MPA1446 specific for clone MPA in the family *Syntrophomonadaceae*; (B) mesophilic stearate culture (enrichment MST) hybridized with probe MST445 specific for clone MST in the class *Deltaproteobacteria*; (C) the mesophilic oleate culture (enrichment MOL) hybridized with probe MSP1446 specific for clones MOL and MLI in the family *Syntrophomonadaceae*; (D) mesophilic linoleate culture (enrichment MLI) hybridized with probe MSP1446; (E) thermophilic palmitate culture (enrichment TPA) hybridized with probe TSP436 specific for clones TPA, TST, and JA2 in the family *Syntrophomonadaceae*; (F) thermophilic stearate culture (enrichment TST) hybridized with probe TSP436; (G) thermophilic oleate culture (enrichment TOL) hybridized with probe TOL1028 specific for clone TOL in the phylum *Firmicutes*. Bars represent 10 μm .

indicate that the dominant microorganisms in both TOL and MST enrichment cultures incorporate ^{13}C from palmitate, indicating that these microbes could degrade LCFA in enrichment cultures.

Isolation of LCFA degraders in pure culture. Since most LCFA degraders in the family *Syntrophomonadaceae* have been known to utilize butyrate, crotonate, and butyrate plus pentenoate as alternative energy sources (5, 54), we first attempted to isolate LCFA oxidizers from two thermophilic enrichment cultures (TPA and TST) and three mesophilic enrichment cultures (MPA, MOL, and MLI) using serial dilution or roll-tube isolation with these substrates. However, the first trial met with failure due to the outgrowth of nontarget microbes, which were *Syntrophomonas* spp. or *Syntrophothermus* spp. by analysis of 16S rRNA gene sequences or by FISH with a Synm700 probe (11), could utilize butyrate syntrophically with methanogens but could not degrade LCFA (data not shown). The majority of these cells did not react with specifically designed probes, suggesting that these microbes were not the dominant bacteria in the original enrichments. We therefore serially diluted the enrichments with medium containing *M. hungatei* or *M. thermautotrophicus* type II cultures and supplemented with LCFA to further enrich the LCFA degrader. This process was repeated several times, and the cultures were routinely checked by FISH. After this step, we obtained highly purified cocultures from enrichment cultures MPA and MLI with *M. hungatei* and from TPA with *M. thermautotrophicus* type II. Again, to isolate the LCFA-degrading bacteria in pure culture, we used roll-tube isolation with medium containing 10 mM crotonate or 10 mM butyrate plus 10 mM pentenoate (53) as a substrate and supplemented with 4 mM 2-bromoethane sulfonate to inhibit the methanogens. After repeating this step several times, we successfully isolated strain MPA in pure culture (from enrichment culture MPA). The cells were slightly curved rods and 1.5 to 4.0 μm long and 0.4 to 0.6 μm wide, morphologically similar to other *Syntrophomonas* spp. Strain MPA could degrade palmitate in coculture with *M. hungatei*. We also obtained a highly purified culture of TPA (from enrichment culture TPA), which still included a few *Thermodesulfobivrio* cells. TPA cocultured with *M. thermautotrophicus* type II could degrade palmitate, too (data not shown).

We attempted to isolate LCFA oxidizers from enrichment culture MST, but we were unable to do so due to the outgrowth of *Syntrophomonas* spp. as described above. Next, we tried to isolate the MST445-positive cells using sulfate or sulfur as an electron acceptor and stearate or butyrate as an electron donor, since the dominant bacterium belongs to the class *Deltaproteobacteria*, many members of which are sulfate- and sulfur-reducing bacteria (16). This approach supported the growth of only vibrioid-type cells (later, we identified the cells as *Desulfobivrio* spp. [data not shown]), not MST445 probe-positive bacteria. We could not find any suitable substrate other than LCFA (palmitate, stearate, and oleate), and hence, we were not able to isolate the dominant bacterial cells in enrichment culture MST even in coculture with methanogens.

Since the dominant bacteria were related to the anaerobic ethanol-oxidizing syntroph *T. syntrophicus*, which could grow in pure culture, mineralizing sucrose as a substrate (40), we used sucrose to isolate the target cells from enrichment culture

TOL. After repeated roll-tube isolation, a pure culture of strain TOL was obtained. However, when we incubated strain TOL with *M. thermautotrophicus* strain type II, oleate degradation was not observed in over 6 months of incubation. We also tried to cultivate strain TOL with palmitate, oleate, and stearate under different conditions (for example, by replacing a hydrogen-scavenging partner, *M. thermautotrophicus* strain type II, with *T. yellowstonii* and establishing a triculture with hydrogenotrophic methanogens and acetate utilizing *M. thermophila* and supplementing with a supernatant of filter-sterilized enrichment culture TOL), but we were unable to demonstrate LCFA degradation.

DISCUSSION

Physiological characteristics of LCFA-degrading enrichment cultures. Syntrophic LCFA-degrading methane-producing enrichment cultures were sometimes unstable. This phenomenon has been observed before, not only in LCFA enrichments (4) but also in other enrichment cultures involving syntrophic interactions with methanogens (12, 34). As noted in our experiments, enrichment cultures MLI and TLI, which were grown on linoleate as a substrate, were extremely unstable and TLI could not be grown after three successive transfers even when a large inoculum was used (25% [vol/vol]). Previous reports have suggested that longer-chain and more-unsaturated LCFA are more toxic to microorganisms (20, 21). This means that linoleate is the most toxic of the four LCFA substrates (palmitate, stearate, oleate, and linoleate) used in this study. We therefore believe that the stability and cultivability issues observed for enrichment culture TLI may be due to both the LCFA toxicity and the complexity inherent in handling anaerobic syntrophic consortia that require hydrogen transfer to methanogens. This is one reason for the difficulty in isolating LCFA-degrading syntrophs.

The degradation patterns of LCFA in our enrichment cultures were in accordance with previous reports (Table 2) (4, 32), and so the mechanism of LCFA degradation was assumed to be β -oxidation. The specific growth rates of our enrichment cultures were estimated to be 0.06 to 0.8 day^{-1} , comparable to previous reports, e.g., 0.53 and 0.6 day^{-1} for mesophilic oleate- and stearate-degrading enrichment cultures, respectively (35); 0.13 day^{-1} for thermophilic oleate-degrading enrichment culture (32); and 0.3 day^{-1} for thermophilic stearate-degrading enrichment culture (4). This slow growth of LCFA-degrading syntrophs is another reason for the difficulty of isolation. Consequently, in our study, more than 3 years were required to establish highly enriched cultures as well as to isolate the microbes responsible for the degradation of LCFA.

LCFA-degrading members of the family *Syntrophomonadaceae*. Even though we attempted to isolate LCFA-degrading microorganisms by employing a strategy that was successfully applied in our previous studies to isolate anaerobic syntrophic microorganisms (14, 34), the isolation of LCFA-degrading microbes has been difficult. During the isolation procedure, nontarget *Syntrophomonas* spp. or *Syntrophothermus* spp. that could degrade butyrate but not LCFA were frequently isolated or overgrown. These microbes may survive by using short-chain fatty acids leached from LCFA oxidizers during β -oxidation. The family *Syntrophomonadaceae* contains not only

LCFA degraders but also species that utilize short-chain fatty acids but not LCFA (44). All LCFA degraders in this family except *S. curvata* have been isolated via LCFA enrichment culture (23, 36, 46, 52). On the contrary, short-chain fatty acid-oxidizing syntrophs, which cannot utilize LCFA, were enriched and isolated only when butyrate or short-chain fatty acids were used (31, 41, 53–55). This could be a consequence of substrate affinity based on carbon length, and thus, some *Syntrophomonas* spp. or *Syntrophothermus* spp. were overgrown in the isolation procedure in butyrate medium. In addition, substrate specificity also may affect the overgrowth of *Syntrophomonas* spp. or *Syntrophothermus* spp. that cannot degrade LCFA in our experiment. The substrate specificity of β -oxidation has been shown to depend on the carbon chain length specificity of the acyl-coenzyme A dehydrogenase (50). This enzyme catalyzes the energetically most unfavorable reaction in the β -oxidation pathway (39, 50), and forms of this enzyme with correspondingly different substrate specificities are known to exist. Thus, it is thought that each fatty acid-degrading *Syntrophomonas* sp. or *Syntrophothermus* sp. has a preferred fatty acid chain length.

Our isolate strain MPA belongs to the genus *Syntrophomonas* (Fig. 1A) and shares some basic traits with the known species of *Syntrophomonas*, such as morphology, syntrophic growth, and degradation manner of LCFA. However, the 16S rRNA gene sequence similarity is 94%, sufficient to describe a new species. In the near future, more-detailed physiological properties will be reported. We observed apparent LCFA degradation by clone TPA. To our knowledge, there is no isolate of thermophilic, neutrophilic, LCFA-degrading syntrophs to date. The LCFA-degrading bacterium in culture TPA is thought to be a new thermophilic LCFA-degrading member of the family *Syntrophomonadaceae*, but this should be confirmed in pure culture.

LCFA-degrading microorganisms not affiliated with the family *Syntrophomonadaceae*. To our knowledge, *S. aciditrophicus* is the only previously reported LCFA-degrading syntroph not affiliated with the family *Syntrophomonadaceae*, but it is known primarily as a benzoate degrader. Recently, Grabowski et al. also reported that a bacterial clone closely related to the genus *Syntrophus* in the class *Deltaproteobacteria* may be involved in stearate and heptadecanoate degradation (9). Therefore, it is apparent that several types of bacterial groups may be involved in the degradation of LCFA. In enrichment culture MST, FISH and RNA-SIP analyses strongly suggest that the dominant microbes are MST445-positive cells engaged in the degradation of LCFA, but we could not isolate the bacterium. We need further studies to obtain evidence for which specific microbes degrade LCFA in the enrichment culture. On the other hand, we isolated strain TOL, which is expected to metabolize LCFA based on RNA-SIP analysis. However, strain TOL has not shown LCFA degradation ability in artificially constructed cultures like the coculture with *M. thermautotrophicus* strain type II. The reason for this is unclear, but the enrichment culture TOL still contained other microorganisms. It is possible that strain TOL may simply be LCFA tolerant and that these other bacteria may degrade LCFA or be indirectly involved in the degradation of LCFA, but further studies are needed to confirm this.

At present, we are investigating LCFA-degrading microor-

ganisms using RNA-SIP with ^{13}C -labeled palmitate in anaerobic sludges. It is expected that these studies may provide us with more-comprehensive information about the diversity of LCFA-degrading microorganisms under methanogenic conditions.

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