APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2007, p. 1054–1064 Vol. 73, No. 4
0099-2240/07/$08.00+0 doi:10.1128/AEM.01723-06

Microbial Communities Involved in Anaerobic Degradation of Unsaturated or Saturated Long-Chain Fatty Acids▽†

Diana Z. Sousa,[1,2] M. Alcina Pereira,[1,2] Alfons J. M. Stams,[2] M. Madalena Alves,[1]* and Hauke Smidt[2]

Center of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal,[1] and Laboratory of Microbiology, Wageningen University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands[2]

Received 21 July 2006/Accepted 1 December 2006

Anaerobic long-chain fatty acid (LCFA)-degrading bacteria were identified by combining selective enrichment studies with molecular approaches. Two distinct enrichment cultures growing on unsaturated and saturated LCFAs were obtained by successive transfers in medium containing oleate and palmitate, respectively, as the sole carbon and energy sources. Changes in the microbial composition during enrichment were analyzed by denaturing gradient gel electrophoresis (DGGE) profiling of PCR-amplified 16S rRNA gene fragments. Prominent DGGE bands of the enrichment cultures were identified by 16S rRNA gene sequencing. A significant part of the retrieved 16S rRNA gene sequences was most similar to those of uncultured bacteria. Bacteria corresponding to predominant DGGE bands in oleate and palmitate enrichment cultures clustered with fatty acid-oxidizing bacteria within *Syntrophomonadaceae* and *Syntrophobacteraceae* families. A low methane yield, corresponding to 9 to 18% of the theoretical value, was observed in the oleate enrichment, and acetate, produced according to the expected stoichiometry, was not further converted to methane. In the palmitate enrichment culture, the acetate produced was completely mineralized and a methane yield of 48 to 70% was achieved from palmitate degradation. Furthermore, the oleate enrichment culture was able to use palmitate without detectable changes in the DGGE profile. However, the palmitate-specialized consortia degraded oleate only after a lag phase of 3 months, after which the DGGE profile had changed. Two predominant bands appeared, and sequence analysis showed affiliation with the *Syntrophomonas* genus. These bands were also present in the oleate enrichment culture, suggesting that these bacteria are directly involved in oleate degradation, emphasizing possible differences between the degradation of unsaturated and saturated LCFAs.

Long-chain fatty acids (LCFA) are the main products of lipid hydrolysis and are frequently found in wastewaters from various sources, e.g., dairy industry, food processing industry, slaughterhouses, wool scouring industry, and vegetable oil/fat refineries. LCFA vary in chain length and degree of saturation, and the most abundant saturated and unsaturated LCFA present in wastewaters are palmitate ($C_{16:0}$) and oleate ($C_{18:1}$), respectively (27, 42). Palmitate is also a key intermediate of oleate degradation (24). Although anaerobic hydrolysis of lipids to glycerol and LCFA occurs rapidly, subsequent LCFA degradation via β-oxidation proceeds rather slowly (23). Proton-reducing acetogens degrade LCFA in syntrophic association with hydrogen-utilizing methanogens and acetoclastic methanogens (33). LCFA oxidation to acetate is possible only when the hydrogen partial pressure in the medium is kept low and, therefore, cooperation with hydrogen-consuming microorganisms is necessary (Table 1).

To date, 10 bacterial species and/or subspecies have been identified that are capable of degrading anaerobically fatty acids with more than 4 carbon atoms and up to 18 carbon atoms. All of these bacteria belong to the families *Syntrophomonadaceae* (21, 44, 50) and *Syntrophaceae* (13) and function together with methanogens during fatty acid degradation. LCFA with more than 12 carbon atoms are utilized by *Syntrophomonas sapovorans* (29), *Syntrophomonas wolfei* subsp. *saponavida* (18), *Syntrophomonas curvata* (49), *Thermosyntropha lipolytica* (40), and *Syntrophus aciditrophicus* (13). Among these microorganisms, the capability of utilizing mono- and/or polyunsaturated LCFA (with more than 12 carbon atoms) is restricted to *Syntrophomonas sapovorans* (29), *Syntrophomonas curvata* (49), and *Thermosyntropha lipolytica* (40). It is, however, still unknown why only certain microorganisms can degrade unsaturated fatty acids in addition to saturated LCFA. In fact, description of the sequence of reactions involved in unsaturated LCFA degradation is still ambiguous. Weng and Jeris (43) suggested that the degradation of unsaturated LCFA starts with chain saturation, whereas other authors provide evidence that direct β-oxidation of unsaturated LCFA is feasible (14, 16, 29). Presently, it is not known whether saturated and unsaturated LCFA are degraded by the same bacteria in methanogenic environments. Investigation of the microbial population dynamics in the presence of unsaturated and saturated LCFA will expand our knowledge of the microbial consortia involved in the process. This information might be essential for the design of better anaerobic treatment processes, in terms of LCFA degradation capacity and enhancement of biogas production from lipids. The use of molecular methods has been shown to add more detailed information about the genetic diversity (3). Fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE) of PCR-amplified fragments, provide an estimation of genetic diversity and can be used to monitor the succession in microbial communities as well as the progress of enrichment cultivation (9, 26, 28, 32). Furthermore, cloning and sequence analysis allow for the iden-

* Corresponding author. Mailing address: Center of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. Phone: 351.253604402. Fax: 351.253678986. E-mail: madalena.alves@deb.uminho.pt.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 8 December 2006.

TABLE 1. Gibbs free energy changes at 25°C for the (possible) reactions involved in syntrophic conversion of oleate and palmitate during methanogenic decomposition[a]

Reaction no.	Process	Reaction	$\Delta G^{\circ\prime}$ (kJ reaction^{-1})[b]	$\Delta G'$ (kJ reaction^{-1})[c]
1	Oleate degradation	$C_{18}H_{33}O_2^- + 16H_2O \rightarrow 9C_2H_3O_2^- + 15H_2 + 8H^+$	+391	−131
2	Palmitate degradation	$C_{16}H_{31}O_2^- + 14H_2O \rightarrow 8C_2H_3O_2^- + 14H_2 + 7H^+$	+419	−81
3	Hydrogenotrophic methanogenesis	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−136	−20
4	Acetoclastic methanogenesis	$C_2H_3O_2^- + H_2O \rightarrow HCO_3^- + CH_4$	−31	−19

[a] Data were obtained or calculated from data reported by Thauer et al. (41) and Lalman (15).
[b] Gibbs free energies (at 25°C) calculated under standard conditions (solute concentrations of 1 M and gas partial pressure of 10^5 Pa).
[c] Gibbs free energies (at 25°C) for oleate/palmitate concentration of 1 mM, acetate concentration of 10 mM, and H_2 partial pressure of 1 Pa.

tification of predominant microorganisms present in such complex consortia (26, 28).

In this work, we used a combination of cultivation techniques with molecular tools, such as 16S rRNA gene-targeted PCR, DGGE, and cloning and sequencing, to study and compare, for the first time, the microbial communities involved in the degradation of unsaturated and saturated LCFA. Two distinct enrichment series in oleate (unsaturated, $C_{18:1}$)- and palmitate (saturated, $C_{16:0}$)-containing media were made by successive transfers. Consortium enrichment was followed by DGGE fingerprinting, and the predominant microorganisms present in the stable consortia were identified by 16S rRNA gene sequencing. Simultaneously, physiological differences between the microorganisms involved in unsaturated and saturated LCFA degradation were investigated.

MATERIALS AND METHODS

Source of sludge sample and microorganisms for enrichment cultures. Anaerobic sludge, previously acclimated to LCFA, was used as seed sludge for starting the enrichment cultures. Sludge acclimation consisted of a two-step process. First, a continuous loading with skim milk-oleate- and oleate-based synthetic wastewater was carried out in an expanded granular sludge bed (EGSB) reactor, followed by incubation of the LCFA-loaded sludge in batch vials for degradation of the accumulated substrate. The 10-liter EGSB reactor was inoculated with 5 liters of suspended sludge (12.57 g volatile suspended solids liter^{-1}), obtained from a local municipal sludge digester (Freixo ETAR, Porto, Portugal). During the first 64 days of operation, the reactor was fed with a mixture of skim milk and sodium oleate at concentrations between 1 and 4 g chemical oxygen demand (COD) liter^{-1}, with skim milk representing a fraction of 50 to 25% of the total COD. During this period, the hydraulic retention time was gradually decreased from 3 days to 1 day. From day 64 onwards, sodium oleate became the sole carbon source fed at a concentration of 4 g COD liter^{-1} and at a constant hydraulic retention time of 1 day. Macro- and micronutrients were added to the feed according to its composition as described elsewhere (2). To achieve suitable alkalinity, 5 g $NaHCO_3$ was added per liter of feed. At day 77 of operation, the sludge exhibited a whitish and greasy appearance attributed to the accumulation of LCFA on the biomass (25). This sludge was collected from the EGSB reactor and subsequently washed and centrifuged (1,681 × *g*, 10 min) twice with anaerobic basal medium. The basal medium was prepared under strict anaerobic conditions using demineralized water and contained 0.5 g liter^{-1} cysteine-HCl and 3 g liter^{-1} sodium bicarbonate. The final pH of the medium was adjusted to 7.0 to 7.2 with 8 N NaOH. The LCFA-loaded sludge was then incubated in anaerobic batch assays with the same anaerobic basal medium. Incubation was done at 37°C, with stirring at 150 rpm, to allow degradation of the biomass-associated LCFA. This process lasted approximately 10 days, after which methane production ceased. This LCFA-depleted sludge was used to inoculate 50 ml of sterile, anaerobic mineral medium to a final concentration of 8% (vol/vol). Oleate and palmitate enrichments were started by adding the respective substrate to the serum bottles.

Methanospirillum hungatei DSM 864^T (10) and *Methanobacterium formicicum* DSM 1535^T (6) were kindly provided by Caroline Plugge, Laboratory of Microbiology, Wageningen University, The Netherlands.

Medium composition and cultivation. A bicarbonate-buffered mineral salt medium (36) was used to grow the enrichment cultures and microorganisms. The serum bottles were sealed with butyl rubber septa and aluminum crimp caps, and the headspace was flushed with a mixture of N_2-CO_2 (80:20; 1.7 × 10^5 Pa). For cultivation of *M. hungatei* DSM 864^T and *M. formicicum* DSM 1535^T, a mixture of H_2-CO_2 (80:20; 1.25 × 10^5 Pa) was used instead. Mineral medium was reduced with 0.8 mM sodium sulfide ($Na_2S \cdot 9H_2O$) before inoculation. Substrates were supplied by adding sterile stock solutions to the medium to obtain the required final concentrations. All inoculations and transfers were done aseptically using sterile syringes and needles, and all cultures were incubated statically at 37°C in the dark.

Enrichment cultures. Two distinct enrichment series were obtained by successive transfers of active cultures (10%) into new medium containing oleate ($C_{18:1}$, unsaturated LCFA) and palmitate ($C_{16:0}$, saturated LCFA), respectively, as the sole carbon and energy sources. Oleate and palmitate (sodium salts of fatty acids) (Fluka, Riedel-de-Haën, and Sigma-Aldrich, Seelze, Germany) were added to the medium from sterile stock solutions to a final concentration of 1 mM. Five percent (vol/vol) of an active culture of *M. hungatei* DSM 864^T was added to the subcultures as a hydrogen scavenger. In the oleate enrichment culture, the added *M. hungatei* DSM 864^T strain did not show active growth (verified by microscopy observation and archaeon-specific 16S rRNA gene-targeted DGGE fingerprinting). Therefore, *M. formicicum* DSM 1535^T was used as a hydrogen-consuming partner in the oleate enrichment cultures. LCFA degradation was indirectly monitored by measuring methane concentration in the headspace of the serum bottles. Methane yield in stable oleate and palmitate enrichment cultures was calculated as the ratio of the mass of methane present in bottle headspace to the theoretical mass of methane that would be obtained from the complete degradation of 1 mM oleate or palmitate.

Substrate utilization tests. The ability of oleate and palmitate enrichment cultures to metabolize different saturated and unsaturated fatty acids (C_2 to C_{18}) was studied in the bicarbonate-buffered medium used for the enrichment cultures. Substrates were added from sterile anaerobic concentrated stock solutions to final concentrations of 10 mM (C_2 to C_4), 5 mM (C_5 to C_9), and 1 mM (C_{10} to C_{18}). Five percent of an active culture of *M. formicicum* DSM 1535^T or *M. hungatei* DSM 864^T was added to the subculture of the oleate or palmitate enrichment culture, respectively. LCFA degradation was estimated by measuring product formation, i.e., methane and volatile fatty acids (VFA). Liquid samples (0.5 ml) were withdrawn from the bottles for VFA analysis. Methane concentrations measured in the headspace of the bottles were corrected for the volumes of samples removed. Recovery of the different fatty acids in the form of acetate, propionate, and methane was calculated using mass balances.

Substrate swap. Oleate and palmitate enrichment cultures, corresponding to early (after 2 successive transfers) and late (after 16 successive transfers) stages of specialization, were submitted to substrate swaps from oleate to palmitate and palmitate to oleate, respectively. Setup and monitoring of cultures were as described for enrichment cultures.

Analytical methods. Gas samples were analyzed by gas chromatography with a GC-14B apparatus (Shimadzu, Kyoto, Japan) equipped with a packed column (Molsieve 13× 60/80 mesh, 2-m length, 2.4-mm internal diameter; Varian, Middelburg, The Netherlands) and a thermal conductivity detector. The oven temperature was 100°C, and the injector and detector temperatures were 90 and 150°C, respectively. Argon was the carrier gas at a flow rate of 30 ml min^{-1}. VFA were analyzed by high-pressure liquid chromatography from centrifuged (10,000 × *g*, 10 min) samples of the culture media. VFA were measured with a Polyspher OA HY column (300 by 6.5 mm; Merck, Darmstadt, Germany) and an RI SE-61 refractive index detector (Shodex, Tokyo, Japan). The mobile phase

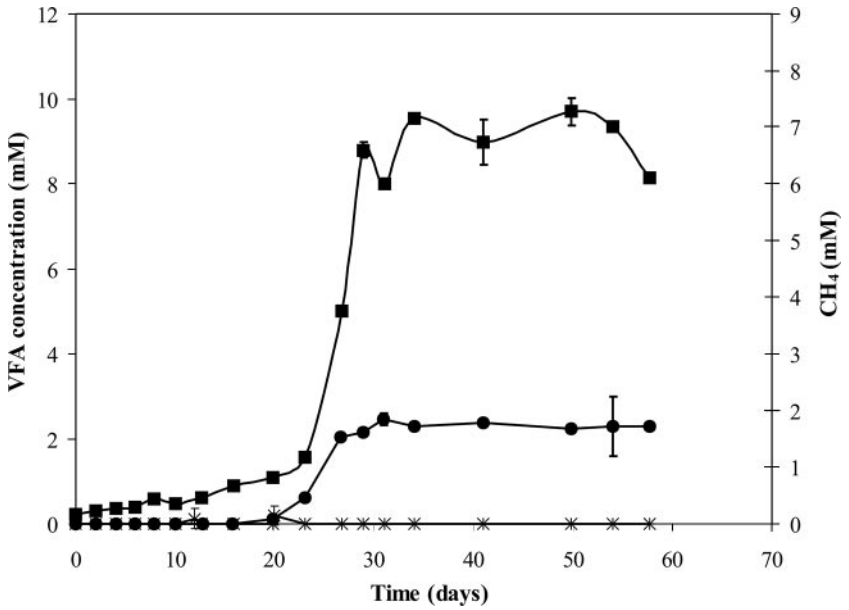


FIG. 1. Methane and VFA production during oleate degradation by a stable oleate enrichment culture after 21 successive transfers (OM21). ■, acetate; ×, butyrate; ●, methane. The bars indicate maximum and minimum values measured at each time point ($n = 2$).

was 0.01 N H_2SO_4 at a flow rate of 0.6 ml min^{-1}. The column temperature was 60°C.

DNA extraction and PCR amplification. Approximately 10-ml aliquots of well-homogenized microbial cultures were concentrated by centrifugation (10,509 × *g*, 5 min), immediately frozen at the time of sampling, and stored at −20°C. Total genomic DNA was extracted using a FastDNA SPIN kit for soil (Qbiogene, Carlsbad, CA).

The 16S rRNA genes were amplified by PCR using a *Taq* DNA polymerase kit (Life Technologies, Gaithersburg, MD) with primers targeting conserved domains. All primers used were synthesized commercially by MWG Biotech (Ebersberg, Germany) (see Table S1 in the supplemental material). Bacterial 16S rRNA genes were selectively amplified for cloning using primers Bact27-f and Uni1492-r. The thermocycling program used for amplification was as follows: predenaturation at 95°C for 5 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 40 s, and elongation at 72°C for 90 s; and postelongation at 72°C for 5 min. The reactions were subsequently stopped by cooling to 4°C. For DGGE analysis, PCR products were generated using primers U968GC-f and L1401-r, targeting the V6 to V8 regions of bacterial 16S rRNA. The program for amplification was as described above but with 35 cycles and an annealing temperature of 56°C. The size and yield of PCR products were estimated by 1% (wt/vol) agarose gel electrophoresis and ethidium bromide staining by using a 100-bp DNA ladder (MBI Fermentas, Vilnius, Lithuania).

DGGE analysis of PCR amplicons. DGGE analysis of the amplicons was done as described by Zoetendal et al. (51) by using the DCode system (Bio-Rad, Hercules, CA) with 8% (vol/vol) polyacrylamide gels and a denaturant gradient of 30 to 60%. A 100% denaturing solution was defined as 7 M urea and 40% formamide. Electrophoresis was performed for 16 h at 85 V in a 0.5× Tris-acetate-EDTA buffer at 60°C. DGGE gels were stained with $AgNO_3$ as described by Sanguinetti et al. (31). Gels were scanned at 400 dots per inch and analyzed using the GelCompar II software package, version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). After normalization, bands were defined for each sample by using the band search algorithm within the program and band density-based dendrograms were generated. Similarity between DGGE profiles was determined by calculating similarity indices of the densitometric curves of the profiles compared using the Pearson product-moment correlation (12). Peak heights in the densitometric curves were used to determine the diversity indices based on the Shannon-Weiner diversity index, calculated as $H = -\Sigma[P_i \ln(P_i)]$, where H is the diversity index and P_i is the importance probability of the bands in a lane ($P_i = n_i/n$, where n_i is the height of an individual peak and n is the sum of all peak heights in the densitometric curves).

Cloning and sequencing of PCR-amplified products. PCR amplicons were purified with a QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions and cloned into *Escherichia coli* JM109 (Invitrogen, Breda, The Netherlands) by using the Promega pGEM-T Easy vector system (Promega, Madison, WI). Insert size was confirmed by PCR amplification with the pGEM-T-specific primers PG1-f and PG2-r. Amplicons of the correct size were screened by amplified ribosomal DNA restriction analysis (ARDRA), using the restriction enzymes MspI, CfoI, and AluI (Promega, Madison, WI); acetylated bovine serum albumin was added to the digestion reaction to a final concentration of 0.1 mg ml^{-1}. The restriction fragments were analyzed by electrophoresis in a 4% (wt/vol) agarose gel and visualized with ethidium bromide. Plasmids of selected transformants, with different ARDRA patterns and corresponding to predominant bands in the DGGE community fingerprint, were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and subjected to DNA sequence analysis. Sequencing reactions were performed at BaseClear (Leiden, The Netherlands) using pGEM-T vector-targeted sequencing primers SP6 and T7 and 16S rRNA gene-targeted internal primers Uni-533-forw and Bact-1100-rev for bacterial 16S rRNA gene amplification. The sequences were assembled and aligned using the programs EditSeq and SeqMan II (DNAStar, Madison, WI). The consensus sequences obtained were checked for potential chimera artifacts by the CHECK_CHIMERA program of Ribosomal Database Project II (RDP release 8.1, http://rdp8.cme.msu.edu/html/) (8).

Phylogenetic placement. Similarity searches for the 16S rRNA gene sequences derived from the sludge clones were performed using the NCBI BLAST search program within the GenBank database (1). Alignment of the 16S rRNA sequences was performed by using the FastAligner v1.03 tool of the ARB program package (19). The resulting alignments were manually checked and corrected when necessary, and unambiguously aligned nucleotide positions were used for the construction of a 16S rRNA gene-based phylogenetic tree by using the neighbor-joining method (30). Phylogenetic placement was performed in comparison with reference sequences, with Felsenstein correction and application of appropriated filters at the respective phylum level.

Nucleotide sequence accession numbers. Nearly complete 16S rRNA sequences of the 16S rRNA gene clones have been deposited in the GenBank database under accession numbers DQ459209 to DQ459216.

RESULTS

Description of the methanogenic oleate and palmitate enrichment cultures. Two distinct oleate and palmitate enrichment cultures were obtained through numerous transfers into fresh medium containing oleate and palmitate (1 mM), respectively. Incubation was performed at 37°C, and no terminal

TABLE 2. Fermentation balances of the oleate enrichment culture with various organic acids as substrates[a]

Substrate	No. of carbon atoms: no. of double bonds	Initial substrate concn (mM)	Concn after incubation (mmol liter^{-1} medium)[b]				Mass balance recovery (%)[c]
			Acetate	Propionate	Butyrate	Methane	
Acetate	2:0	10	10.8				0
Propionate	3:0	10		10.1			0
Butyrate	4:0	10	11.4		4.8	1.7	56
i-Butyrate	4:0	10					0
Valerate	5:0	5	5.0	4.6		1.6	94
i-Valerate	5:0	5					0
Caproate	6:0	5	15.1		0.3	3.4	101
i-Caproate	6:0	5					0
Enanthate	7:0	5	10.4	4.7		2.8	97
Caprylate	8:0	5	21.2		0.1	4.6	103
Pelargonate	9:0	5	15.4	4.8		2.8	95
Caprate	10:0	1	5.2			0.7	98
Hendecanoate	11:0	1	4.0	0.6		0.8	86
Laureate	12:0	1	6.3			1.9	103
Myristate	14:0	1	6.9			2.0	95
Valerenate	15:0	1	5.5	0.5		1.7	82
Palmitate	16:0	1	7.8			1.3	92
Palmitoleate	16:1	1	0.1				1
Margerate	17:0	1	6.1	0.5		1.7	78
Stearate	18:0	1	8.4			2.4	90
Oleate	18:1	1	8.6			1.7	90
Linoleate	18:2	1	0.2				2

[a] Subcultures of the oleate enrichment culture OM21 were used in this assay.
[b] The values shown are means for duplicate cultures corrected for controls with no substrate added (measured after 54 days of incubation).
[c] Calculated with the fermentation products.

electron acceptor was provided, resulting in methanogenic conversion. Due to their low growth rates, oleate and palmitate enrichment cultures could be transferred approximately once a month throughout a 2-year period. Substrate degradation by the enrichment cultures was easily observed by the clearing of oleate or palmitate in solution and/or suspension (addition of oleate and palmitate to the medium causes an immediate turbid/white appearance). After 2 to 3 weeks of incubation, the turbidity decreased and gas production started.

Oleate enrichment culture. The degradation of oleate by a stable oleate enrichment culture led to the accumulation of acetate, which was not converted to methane and carbon dioxide during the course of the experiment (Fig. 1). Other volatile fatty acids were not found as degradation products. After approximately 1 month of incubation, about 97% of the initial substrate could be accounted for by the products measured, i.e., acetate and methane, with average concentrations of 9.2 and 1.8 mmol per liter medium, respectively. Overall, a low methane yield was observed, i.e., 9 to 18%, which reflects the high level of acetate that was not degraded further.

Besides oleate, a number of other fatty acids were degraded by the oleate enrichment culture (Table 2). The enrichment culture produced acetate and methane from linear saturated fatty acids with an even number (4 to 18) of carbon atoms and acetate, propionate, and methane from those with an odd number (5 to 17) of carbon atoms. During the degradation of even-numbered fatty acids, butyrate was occasionally detected as an intermediate. The unsaturated fatty acids palmitoleate and linoleate were converted only to a minor extent, as only a slight acetate accumulation with no methane production was observed for these substrates. Acetate and propionate produced during fatty acid degradation were not further converted to methane and carbon dioxide by the oleate enrichment culture. Branched-chain organic acids, such as *i*-butyrate, *i*-valerate, and *i*-caproate, were not degraded by the oleate enrichment culture.

Palmitate enrichment culture. During long-term incubation of the palmitate enrichment culture with palmitate, accumulation of acetate in the medium started after about 25 days (Fig. 2). From approximately 29 days of incubation onwards, the acetate concentration decreased, which coincided with the onset of methane production. Final methane accumulated to a concentration of 5.6 to 8.0 mmol per liter medium, which corresponds to 48 to 70% of the stoichiometric value expected for complete palmitate degradation. No other VFA were detected during palmitate degradation by the enrichment culture.

The palmitate enrichment culture could use, besides palmitate, a number of other fatty acids (Table 3). Fatty acids with an even number (4 to 18) of carbon atoms were degraded to methane and carbon dioxide, while acids with an odd number (5 to 17) of carbon atoms were also converted to propionate, in addition to methane and carbon dioxide. None of the unsaturated fatty acids tested (palmitoleate, oleate, and linoleate) could be degraded by the palmitate enrichment culture during the course of the experiment. Contrary to the oleate enrichment culture, the palmitate enrichment culture used acetate. However, propionate was not converted to methane and carbon dioxide by this enrichment culture. As in the case of the oleate enrichment culture, branched-chain organic acids, such as *i*-butyrate, *i*-valerate, and *i*-caproate, were not degraded by the palmitate enrichment culture.

Molecular characterization of the oleate and palmitate enrichment cultures. Microbial diversity and shifts in bacterial communities during the selective enrichment on oleate or palmitate were monitored by DGGE fingerprinting of PCR-amplified 16S rRNA gene fragments (Fig. 3 and 4). Phyloge-

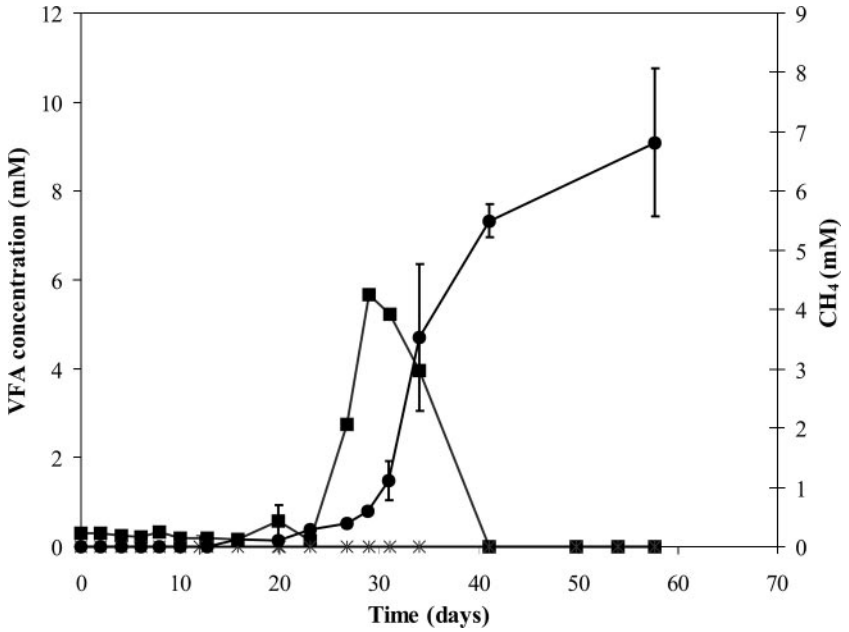


FIG. 2. Methane and VFA production during palmitate degradation by a stable palmitate enrichment culture after 19 successive transfers (PM19). ■, acetate; ×, butyrate; ●, methane. The bars indicate maximum and minimum values measured at each time point ($n = 2$).

netic analysis of the predominant bacterial community, as visualized in the DGGE patterns of stabilized enrichment cultures, was accomplished after 16S rRNA gene cloning and sequence analysis (Fig. 5).

Shannon diversity indices (H) calculated based on DGGE patterns obtained for each of the enrichment samples evidenced a decrease in bacterial diversity over time, as expected (Fig. 3 and 4). In both enrichment series, less complex DGGE patterns were observed after just two to three successive transfers, with a reduction in the number of predominant bands, indicating a rapid reduction in the bacterial richness. For the oleate enrichment series, a considerable decrease in the bacterial richness was already verified during the first incubation with oleate (Fig. 3, lane OM), compared to the inoculum (Fig.

TABLE 3. Fermentation balances of the palmitate enrichment culture with various organic acids as substrates[a]

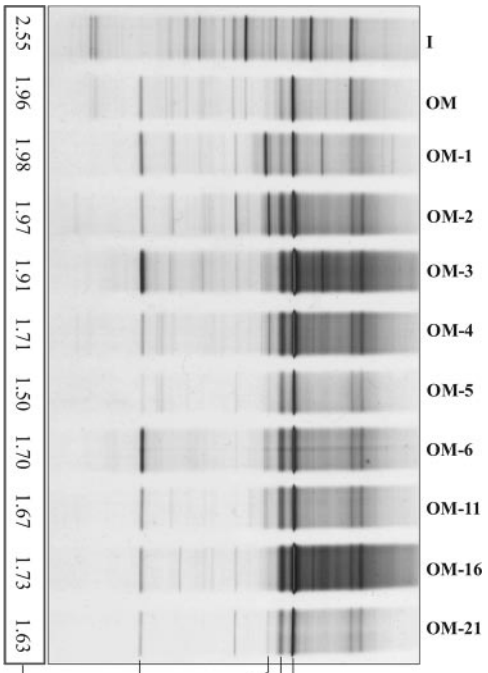
Substrate	No. of carbon atoms: no. of double bonds	Initial substrate concn (mM)	Concn after incubation (mmol liter^{-1} medium)[b]				Mass balance recovery (%)[c]
			Acetate	Propionate	Butyrate	Methane	
Acetate	2:0	10	5.2			3.3	33
Propionate	3:0	10		9.8			0
Butyrate	4:0	10	0.5		0.7	16.7	88
i-Butyrate	4:0	10					0
Valerate	5:0	5	0.3			0.2	2
i-Valerate	5:0	5					0
Caproate	6:0	5				7.8	39
i-Caproate	6:0	5					0
Enanthate	7:0	5	0.3	2.9		10.4	66
Caprylate	8:0	5				19.0	69
Pelargonate	9:0	5	0.2			0.1	2
Caprate	10:0	1	0.3			5.8	99
Hendecanoate	11:0	1	0.4	0.7		0.8	43
Laureate	12:0	1				6.1	71
Myristate	14:0	1				3.3	33
Valerenate	15:0	1		0.6		5.1	58
Palmitate	16:0	1				8.1	70
Palmitoleate	16:1	1					0
Margerate	17:0	1		0.5		3.8	40
Stearate	18:0	1				7.7	59
Oleate	18:1	1					0
Linoleate	18:2	1					0

[a] Subcultures of the palmitate enrichment culture PM19 were used in this assay.
[b] The values shown are means for duplicate cultures corrected for controls with no substrate added (measured after 54 days of incubation).
[c] Calculated with the fermentation products.

3, lane I). From the fourth successive transfer onwards (Fig. 3, lanes OM-4 to OM-21), the oleate enrichment culture showed a nearly stable bacterial composition (Pearson similarity of 82% ± 8% [mean ± standard deviation]) with highly similar diversity (*H* value of 1.66 ± 0.08) and with a few DGGE bands prevailing as relatively more abundant in all of the enrichment samples. Selective enrichment on palmitate led to the development of a seemingly stable bacterial community only after more than six subcultures (Pearson similarity of 82% ± 6% and *H* value of 1.73 ± 0.18) (Fig. 4, lanes PM-9 to PM-19). A similar reduction in the bacterial diversity was observed after enrichment in oleate and palmitate, compared with that in the inoculum sludge, with bacterial diversities corresponding to 65% ± 3% and 66% ± 7%, respectively, of the total initial diversity. Although the same inoculum was used for starting up the oleate and palmitate enrichment series, stable DGGE profiles obtained after enrichment in the two substrates were clearly different (Fig. 3 and 4). Predominant bands in DGGE profiles also corresponded to predominant ribotypes observed in the clone libraries (based on ARDA screening). BLAST similarity searches showed that the corresponding 16S rRNA gene sequences were most closely affiliated with those of not-yet-cultured microorganisms (Fig. 3 and 4). Phylogenetic analysis revealed that the few predominant bacterial populations present in the stable oleate and palmitate enrichment cultures, as visualized by DGGE analysis, belong to *Firmicutes* and *Proteobacteria* phyla (Fig. 5).

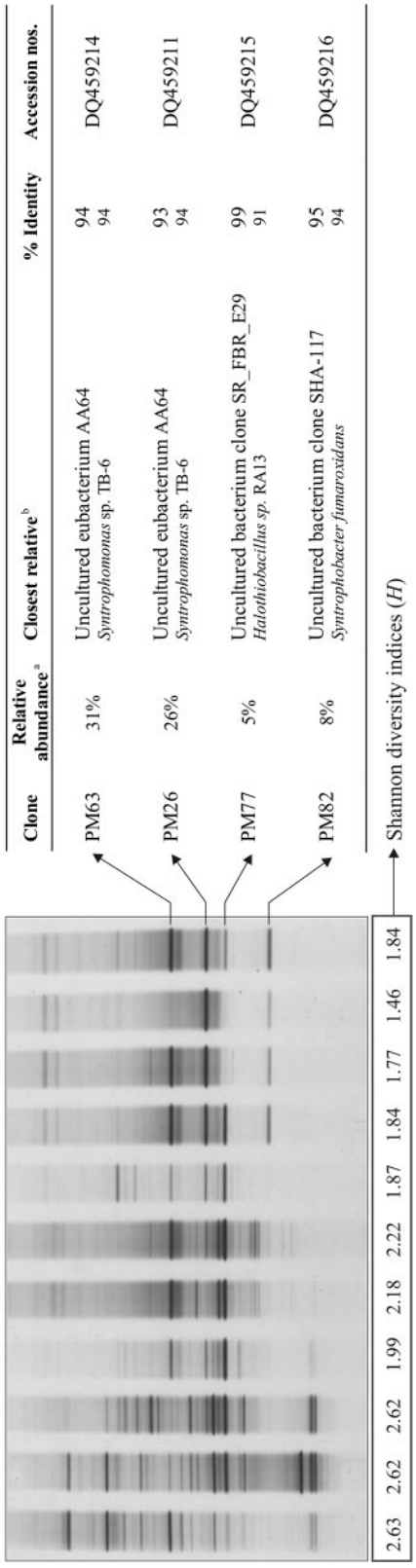
For both oleate and palmitate enrichment cultures, two out of the four predominant DGGE bands identified in each of the stable cultures clustered within the *Syntrophomonas* genus (clones OM14, OM39, PM63, and PM26) (Fig. 3 to 5), indicating the prevalence of syntrophic fatty acid-oxidizing bacteria. The identities of the 16S rRNA gene sequences of clones OM14/OM39 and PM63/PM26 were rather high, i.e., 99% and 97%, respectively. However, the relatedness of clones OM14 and OM39 with clones PM63 and PM26 was only 93%. Additionally, in the palmitate enrichment culture, a predominant DGGE band was identified as a representative of the *Syntrophobacterales* cluster (clone PM82) (Fig. 4 and 5), also a group of bacteria that grow in syntrophic association with methanogens. Despite the absence of sulfate in the medium, a *Desulfovibrio*-like bacterium was detected as a predominant band in the DGGE profile of the oleate enrichment culture (clone OM44) (Fig. 3 and 5). Minor bands clustering within the phyla *Bacteroidetes* and *Proteobacteria*, and with very low identities of 88 and 91% to cultured microorganisms, were identified in the oleate and palmitate enrichments, respectively (clones OM24 and PM77) (Fig. 3 to 5).

Substrate swap. To evaluate the microbial stability of the enrichment cultures obtained on oleate or palmitate in relation to their substrate specificity towards unsaturated or saturated LCFA, substrate swaps from oleate to palmitate or palmitate to oleate, respectively, were performed. These substrate swaps were carried out twice, using enrichment cultures at different stages of enrichment: early cultures, after just two successive transfers (OM2 and PM2), and late cultures, corresponding to stable and highly enriched consortia (OM16 and PM16). Incubation of oleate enrichment cultures (OM2 and OM16) with palmitate led to the production of methane after 20 days (data not shown), which indicated that this substrate could easily be



Clone	Relative abundance[a]	Closest relative[b]	% Identity	Accession nos.
OM14	19%	Uncultured bacterium clone R6b2 *Syntrophomonas* sp. TB-6	94 96	DQ459209
OM39	18%	Uncultured bacterium clone R6b2 *Syntrophomonas* sp. TB-6	94 94	DQ459212
OM24	4%	Uncultured bacterium clone TTA_B21 *Chlorobium phaeobacteroides*	97 88	DQ459210
OM44	6%	*Desulfovibrio aminophilus*	98	DQ459213

FIG. 3. Bacterial DGGE patterns of the oleate enrichment series, obtained by successive transfers in anaerobic media (I, inoculum; OM-x, enrichment culture, where *x* represents the number of transfers), and identification of the predominant DGGE bands present in stable enrichment cultures. Shannon diversity values are presented for each of the analyzed samples. [a], relative abundance of clones with identical ARDRA profiles (calculated from a total of 68 clones containing complete 16S rRNA gene inserts). All of the other ARDRA profiles showed a lower relative abundance (1 to 3%). [b], closest relative species of the selected clones, determined by an NCBI BLAST search. In cases where the first closest database hit was an uncultured microorganism, the first hit of a cultured microorganism is also given.



Clone	Relative abundance[a]	Closest relative[b]	% Identity	Accession nos.
PM63	31%	Uncultured eubacterium AA64	94	DQ459214
		Syntrophomonas sp. TB-6	94	
PM26	26%	Uncultured eubacterium AA64	93	DQ459211
		Syntrophomonas sp. TB-6	94	
PM77	5%	Uncultured bacterium clone SR_FBR_E29	99	DQ459215
		Halothiobacillus sp. RA13	91	
PM82	8%	Uncultured bacterium clone SHA-117	95	DQ459216
		Syntrophobacter fumaroxidans	94	

FIG. 4. Bacterial DGGE patterns of the palmitate enrichment series, obtained by successive transfers in anaerobic media (I, inoculum; PM-*x*, enrichment culture, where *x* represents the number of transfers), and identification of the predominant DGGE bands present in stable enrichment cultures. Shannon diversity values are presented for each of the analyzed samples. [a], relative abundance of clones with identical ARDRA profiles (calculated from a total of 86 clones containing complete 16S rRNA gene inserts). All of the other ARDRA profiles showed a lower relative abundance (1 to 2%). [b], closest relative species of the selected clones, determined by an NCBI BLAST search. In cases where the first closest database hit was an uncultured microorganism, the first hit of a cultured microorganism is also given.

used by the culture in the replacement of oleate. Moreover, these cultures could be successfully subcultured in medium containing palmitate. Conversely, when an early palmitate enrichment culture (PM2) was incubated with oleate, methane production started only after a lag phase of 3 months. Adaptation of the populations to the new substrate might have been a reason for the delay in oleate degradation, also because growth of the following subcultures on oleate was comparatively faster. However, when a late palmitate enrichment culture (PM16) was incubated with oleate, no methane production or growth was observed after more than 6 months of incubation, indicating that the palmitate enrichment culture was no further able to degrade oleate (data not shown).

Figure 6 shows a DGGE fingerprint analysis of the oleate and palmitate enrichment samples submitted for substrate swap. Incubation of the oleate enrichment culture with palmitate did not significantly affect the bacterial DGGE profiles of the cultures (Fig. 6A). This was the case for both the early and late enrichment cultures and subcultures. However, the early palmitate enrichment culture, which degraded oleate only after a lag phase of 3 months, showed clear changes in the DGGE profile after oleate degradation (Fig. 6B). Two predominant bands appeared in the DGGE profile after incubation with oleate (Fig. 6B, lane PM-2→O-1), which remained stable in the subsequent subcultures that grew on oleate (Fig. 6B, lane PM-2→O-8). Comparison of these DGGE profiles with that from a stable oleate enrichment culture showed that the new bands correspond to clones OM14 and OM39, clustering within the *Syntrophomonas* genus. Since the late palmitate enrichment culture did not grow on oleate, no DGGE profiles are available for that culture.

DISCUSSION

Two distinct anaerobic enrichment cultures, growing on oleate or palmitate, were compared by physiological and molecular characterization. These analyses gave insight into the differences in substrate specificity and composition of the microbial consortia involved in the anaerobic degradation of unsaturated and saturated LCFA. In previous studies, several LCFA-degrading bacteria (35) and enrichment cultures (4, 11, 22) were described. However, this is the first report of a comparison between unsaturated and saturated LCFA-degrading enrichment cultures starting from the same inoculum. The highly enriched oleate- and palmitate-degrading cultures were obtained by successive transfers in defined mineral medium. DGGE analysis of stabilized oleate and palmitate enrichment cultures showed different bacterial compositions, although both enrichment cultures were inoculated with the same sludge. Hence, it is possible that different microorganisms in the seed sludge are involved in the degradation of oleate and palmitate. This evidence of differential microbial specializations during oleate and palmitate degradation opens conjectures to differences in the degradation of unsaturated and saturated LCFA. Specificities during the degradation of unsaturated and saturated LCFA were further evidenced by the incubation of stable oleate and palmitate enrichment cultures with different unsaturated LCFA (palmitoleate, oleate, and linoleate). Degradation of oleate by a stable palmitate enrichment culture was not achieved, as was also verified for the

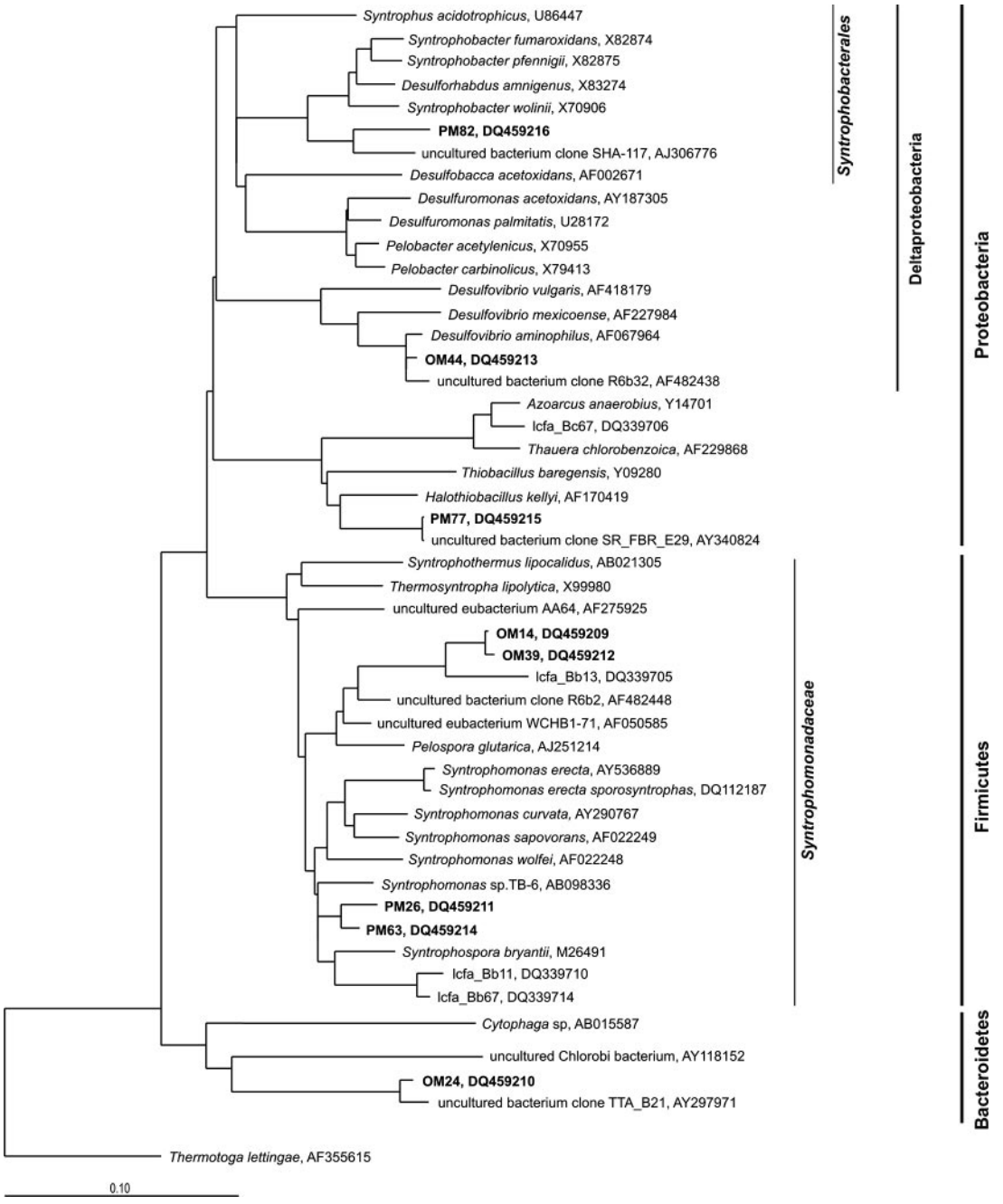


FIG. 5. Phylogenetic tree of bacterial 16S rRNA gene sequences retrieved from the stable oleate and palmitate enrichment cultures. The tree was constructed by using the ARB software package (19) and applying the neighbor-joining method (30). Closely related sequences, with the respective GenBank accession numbers, are shown as references. *Thermotoga lettingae* (AF355615) was used as an outgroup.

other unsaturated LCFA tested (Table 3). The oleate enrichment culture, however, could easily degrade oleate, though degradation of palmitoleate and linoleate was not significant during an extended time of incubation (Table 2).

Analysis of the microbial composition of stable oleate and palmitate enrichment cultures showed that predominant microorganisms, as detected by DGGE analysis, were most closely related to uncultured bacteria, and all belonged to *Firmicutes* and *Proteobacteria* phyla (Fig. 3 to 5). Two predominant bands in each of the enrichment cultures showed affiliation with the *Syntrophomonadaceae* cluster among the branches of fatty acid-oxidizing bacteria (clones OM14, OM39, PM26, and PM63) (Fig. 5). Hence, these microorganisms might play an important role in the degradation of oleate and palmitate by the enrichment cultures. The low 16S rRNA gene sequence identity of these microorganisms with cultured species makes it difficult to speculate about their physiological capabilities, especially with respect to the utilization of LCFA with different levels of chain saturation. However, swapping the substrate of early and late oleate and palmitate enrichment

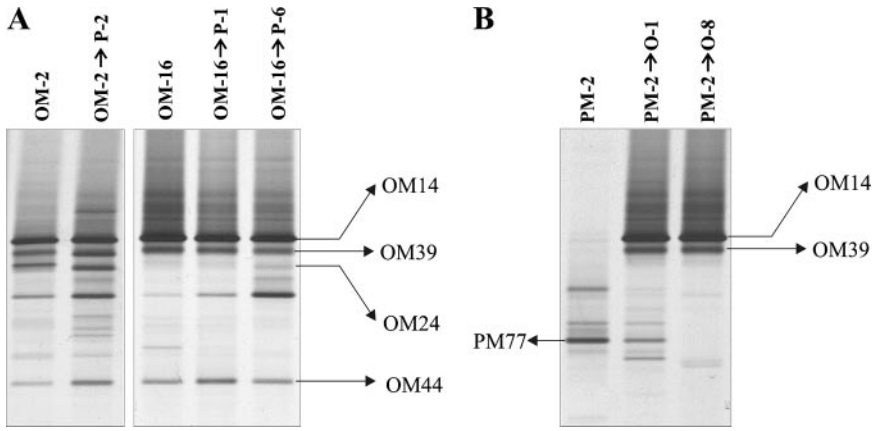


FIG. 6. Bacterial DGGE patterns of the oleate and palmitate enrichment cultures submitted for substrate swap. (A) Results of incubation of early and late oleate enrichment cultures with palmitate and successive transfers. OM-*x*, oleate enrichment culture, where *x* represents the number of transfers; OM-*x*→P-*y*, oleate enrichment culture incubated with palmitate, where *x* and *y* represent the number of transfers. (B) Results of incubation of an early palmitate enrichment culture with oleate and successive transfers. PM-*x*, palmitate enrichment culture, where *x* represents the number of transfers; PM-*x*→O-*y*, palmitate enrichment culture incubated with oleate, where *x* and *y* represent the number of transfers. Arrows indicate DGGE bands identified by 16S rRNA gene sequencing.

cultures for the converse substrate gave insight into the role of bacteria from which clones OM14 and OM39 were derived (Fig. 6B). High identity between 16S rRNA gene sequences of clones OM14 and OM39 (99%) indicated that they most likely represent two strains of the same species, clustering within the *Syntrophomonas* genus. In contrast, sequences of PM26 and PM63 were more distantly related to OM14 and OM39 (93%), suggesting their affiliation to a yet distinct species within the genus (Fig. 5). In fact, an early palmitate enrichment culture (Fig. 6B, lane PM-2) could degrade oleate only after the community was changed, with the clear appearance of two predominant DGGE bands corresponding to clones OM14 and OM39 (Fig. 6B, lane PM-2→O-1). During successive subcultures of this enrichment culture on oleate, DGGE profiles remained stable (Fig. 6B, lane PM-2→O-8). Furthermore, when a late palmitate enrichment culture was incubated with oleate, no degradation occurred after over 6 months of incubation. Conversely, early (Fig. 6A, lane OM-2) and late (Fig. 6A, lane OM-16) oleate enrichment cultures could degrade palmitate without significant changes in the DGGE profiles, which strongly suggests that the microorganisms involved in oleate degradation are also able to degrade palmitate. The fact that palmitate can be used by an oleate enrichment culture with no changes in the microbial community, while oleate cannot be used by the palmitate enrichment culture under the same conditions, suggests that the degradation of unsaturated LCFA involves different enzymes specific to some microorganisms.

Oleate and palmitate enrichment cultures could utilize saturated LCFA (4 to 18 carbon atoms) (Tables 2 and 3). During the β-oxidation of even-numbered fatty acids, acetate was formed as the only organic product. Incubation with odd-numbered fatty acids led to the formation of propionate together with acetate. Propionate was not further degraded by the oleate and palmitate enrichment cultures. This is in accordance with previous reports on syntrophic LCFA degradation by *Syntrophomonadaceae* members (34, 39, 40, 49). Microorganisms belonging to this family were predominant in both enrichment cultures (Fig. 3 to 5). Syntrophic propionate degradation is performed by microorganisms belonging to the genera *Syntrophobacter* (5) and *Smithella* (17). Although the palmitate enrichment culture has one predominant band clustering within the *Syntrophobacter* group (clone PM82) (Fig. 4 and 5), propionate utilization was not observed. However, the identity of the 16S rRNA gene sequences of clone PM82 and *Syntrophobacter fumaroxidans* is very low (94%) and the physiological properties might be different.

Isomers of *n*-fatty acids, such as *i*-butyrate, *i*-valerate, and *i*-caproate, were not degraded by any of the oleate and palmitate enrichment cultures (Tables 2 and 3). Methanogenic degradation of the fatty acid isomers might involve a first step of isomerization that precedes β-oxidation (34, 38, 46). Several methanogenic cocultures have been described as capable of using *i*-butyrate (20, 45), where microorganisms with isomerization and fatty acid-oxidizing capability work together with methanogens. Only two microorganisms were identified that have the capability of performing *i*-butyrate isomerization and β-oxidation together with methanogens, namely, strain IB (46) and *Syntrophothermus lipocalidus* (34). So far, only one defined methanogenic culture has been described that degrades *i*-valerate (37).

Despite the absence of sulfate in the medium, a *Desulfovibrio*-like organism was detected as a predominant band in the DGGE profile of the oleate enrichment culture. In other studies, *Desulfovibrio* species have been detected in methanogenic reactors without added sulfate (7, 47, 48). They were proposed to grow as acetogens in syntrophic association with methanogens. The role of such bacteria in the oleate enrichment culture is not clear yet and needs further investigation.

In conclusion, this study revealed that different microorganisms belonging to the *Syntrophomonadaceae* family were predominant in the oleate and palmitate enrichment cultures but still most closely related to not-yet-cultured microorganisms. Although isolation of pure cultures from syntrophic associations is difficult, due to low growth rates and mutual dependence of partner bacteria, their isolation in pure culture would help to confirm their role in oleate and palmitate degradation.

Moreover, it would be important to get more insight into the differences between unsaturated and saturated LCFA degradation.

ACKNOWLEDGMENTS

We acknowledge Caroline Plugge for the help with Gibbs free energy change calculations.

This study was financed by research grants of the Fundação para a Ciência e Tecnologia (FCT) and Fundo Social Europeu (FSE) attributed to D. Z. Sousa (SFRH/BD/8726/2002) and M. A. Pereira (SFRH/BPD/14591/2003) and a grant of the Wageningen Institute for Environmental and Climate Research (WIMEK).

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Meyers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Alves, M. M., J. A. Vieira, R. M. Pereira, M. A. Pereira, and M. Mota.** 2001. Effect of lipids and oleic acid on biomass development in anaerobic fixed-bed reactors. Part I: biofilm growth and activity. Water Res. **35:**255–263.
3. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
4. **Angelidaki, I., and B. K. Ahring.** 1995. Establishment and characterization of an anaerobic thermophilic (55°C) enrichment culture degrading long-chain fatty acids. Appl. Environ. Microbiol. **61:**2442–2445.
5. **Boone, D. R., and M. P. Bryant.** 1980. Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. Appl. Environ. Microbiol. **40:**626–632.
6. **Bryant, M. P., and D. R. Boone.** 1987. Isolation and characterization of *Methanobacterium formicicum* MF. Int. J. Syst. Bacteriol. **37:**171.
7. **Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. R. Crabill.** 1977. Growth of *Desulfovibrio* in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl. Environ. Microbiol. **33:**1162–1169.
8. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. **31:**442–443.
9. **El-Fantroussi, S., W. Verstraete, and E. M. Top.** 2001. Enrichment and molecular characterization of a bacterial culture that degrades methoxymethyl urea herbicides and their aniline derivatives. Appl. Environ. Microbiol. **67:**4943.
10. **Ferry, J. G., P. H. Smith, and R. S. Wolfe.** 1974. *Methanospirillum*, a new genus of methanogenic bacteria, and characterization of *Methanospirillum hungatei* sp. nov. Int. J. Syst. Bacteriol. **24:**465–469.
11. **Grabowski, A., D. Blanchet, and C. Jeanthon.** 2005. Characterization of long-chain fatty-acid-degrading syntrophic associations from a biodegraded oil reservoir. Res. Microbiol. **156:**814–821.
12. **Hane, B. G., K. Jager, and H. G. Drexler.** 1993. The Pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. Electrophoresis **14:**967–972.
13. **Jackson, B. E., V. K. Bhupathiraju, R. S. Tanner, C. R. Woese, and M. J. McInerney.** 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.
14. **Lalman, J. A., and D. M. Bagley.** 2001. Anaerobic degradation and methanogenic inhibitory effects of oleic and stearic acids. Water Res. **35:**2975–2983.
15. **Lalman, J. A.** 2000. Anaerobic degradation of linoleic (C18:2), oleic (C18:1) and stearic (C18:0) acids and their inhibitory effects on acidogens, acetogens and methanogens. Ph.D. thesis. University of Toronto, Toronto, Canada.
16. **Lalman, J. A., and D. M. Bagley.** 2000. Anaerobic degradation and inhibitory effects of linoleic acid. Water Res. **34:**4220–4228.
17. **Liu, Y. T., D. L. Balkwill, H. C. Aldrich, G. R. Drake, and D. R. Boone.** 1999. Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*. Int. J. Syst. Bacteriol. **49:**545–556.
18. **Lorowitz, W. H., H. X. Zhao, and M. P. Bryant.** 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.
19. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
20. **Matthies, C., and B. Schink.** 1992. Reciprocal isomerization of butyrate and isobutyrate by the strictly anaerobic bacterium strain WoG13 and methanogenic isobutyrate degradation by a defined triculture. Appl. Environ. Microbiol. **58:**1435–1439.
21. **McInerney, M. J.** 1992. The genus *Syntrophomonas*, and other syntrophic bacteria, p. 2048–2057. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes. Springer, New York, NY.
22. **Menes, R. J., A. Fernandez, and L. Muxi.** 2001. Physiological and molecular characterisation of an anaerobic thermophilic oleate-degrading enrichment culture. Anaerobe **7:**17–24.
23. **Pavlostathis, S. G., and E. Giraldogomez.** 1991. Kinetics of anaerobic treatment. Water Sci. Technol. **24:**35–59.
24. **Pereira, M. A., O. C. Pires, M. Mota, and M. M. Alves.** 2002. Anaerobic degradation of oleic acid by suspended and granular sludge: identification of palmitic acid as a key intermediate. Water Sci. Technol. **45:**139–144.
25. **Pereira, M. A., O. C. Pires, M. Mota, and M. M. Alves.** 2005. Anaerobic biodegradation of oleic and palmitic acids: evidence of mass transfer limitations caused by long chain fatty acid accumulation onto the anaerobic sludge. Biotechnol. Bioeng. **92:**15–23.
26. **Pereira, M. A., K. Roes, A. J. M. Stams, M. Mota, M. Alves, and A. D. L. Akkermans.** 2002. Molecular monitoring of microbial diversity in expanded granular sludge bed (EGSB) reactors treating oleic acid. FEMS Microbiol. Ecol. **41:**95–103.
27. **Quemeneur, M., and Y. Marty.** 1994. Fatty-acids and sterols in domestic wastewaters. Water Res. **28:**1217–1226.
28. **Roest, K., M. Altinbas, P. L. Paulo, H. G. H. J. Heilig, A. D. L. Akkermans, H. Smidt, W. M. de Vos, and A. J. M. Stams.** 2005. Enrichment and detection of microorganisms involved in direct and indirect methanogenesis from methanol in an anaerobic thermophilic bioreactor. Microb. Ecol. **50:**440–446.
29. **Roy, F., E. Samain, H. C. Dubourguier, and G. Albagnac.** 1986. *Syntrophomonas sapovorans* sp. nov., a new obligately proton reducing anaerobe oxidizing saturated and unsaturated long-chain fatty acids. Arch. Microbiol. **145:**142–147.
30. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
31. **Sanguinetti, C. J., E. Dias Neto, and A. J. Simpson.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. BioTechniques **17:**914–921.
32. **Santegoeds, C. M., S. C. Nold, and D. M. Ward.** 1996. Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat. Appl. Environ. Microbiol. **62:**3922–3928.
33. **Schink, B.** 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Mol. Biol. Rev. **61:**262–280.
34. **Sekiguchi, Y., Y. Kamagata, K. Nakamura, A. Ohashi, and H. Harada.** 2000. *Syntrophothermus lipocalidus* gen. nov., sp. nov., a novel thermophilic, syntrophic, fatty-acid-oxidizing anaerobe which utilizes isobutyrate. Int. J. Syst. Evol. Microbiol. **50:**771–779.
35. **Sobieraj, M., and D. R. Boone.** 2002. *Syntrophomonadaceae*. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), The prokaryotes: an evolving electronic resource for the microbiological community. Springer-Verlag, New York, NY.
36. **Stams, A. J. M., J. B. van Dijk, C. Dijkema, and C. M. Plugge.** 1993. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl. Environ. Microbiol. **59:**1114–1119.
37. **Stieb, M., and B. Schink.** 1986. Anaerobic degradation of isovalerate by a defined methanogenic coculture. Arch. Microbiol. **144:**291–295.
38. **Stieb, M., and B. Schink.** 1989. Anaerobic degradation of isobutyrate by methanogenic enrichment cultures and by a *Desulfococcus multivorans* strain. Arch. Microbiol. **151:**126–132.
39. **Stieb, M., and B. Schink.** 1985. Anaerobic oxidation of fatty-acids by *Clostridium bryantii* sp. nov., a sporeforming, obligately syntrophic bacterium. Arch. Microbiol. **140:**387–390.
40. **Svetlitshnyi, V., F. Rainey, and J. Wiegel.** 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.
41. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotropic anaerobic bacteria. Bacteriol. Rev. **41:**100–180.
42. **Viswanathan, C. V., B. M. Bai, and S. C. Pillai.** 1962. Fatty matter in aerobic and anaerobic sewage sludges. J. Water Pollut. Control Fed. **34:**189–194.
43. **Weng, C., and J. S. Jeris.** 1976. Biochemical mechanisms in methane fermentation of glutamic and oleic acids. Water Res. **10:**9–18.
44. **Wu, C., X. Liu, and X. Dong.** 2006. *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.
45. **Wu, W. M., M. K. Jain, E. C. de Macario, J. H. Thiele, and J. G. Zeikus.** 1992. Microbial composition and characterization of prevalent methanogens and acetogens isolated from syntrophic methanogenic granules. Appl. Microbiol. Biotechnol. **38:**282–290.

46. **Wu, W.-M., M. K. Jain, and J. G. Zeikus.** 1994. Anaerobic degradation of normal- and branched-chain fatty acids with four or more carbons to methane by a syntrophic methanogenic triculture. Appl. Environ. Microbiol. **60:** 2220–2226.
47. **Zellner, G., P. Vogel, H. Kneifel, and J. Winter.** 1987. Anaerobic digestion of whey and whey permeate with suspended and immobilized complex and defined consortia. Appl. Microbiol. Biotechnol. **27:**306–314.
48. **Zellner, G., and J. Winter.** 1987. Analysis of a highly efficient methanogenic consortium producing biogas from whey. Syst. Appl. Microbiol. **9:**284–292.
49. **Zhang, C. Y., X. L. Liu, and X. Z. Dong.** 2004. *Syntrophomonas curvata* sp. nov., an anaerobe that degrades fatty acids in co-culture with methanogens. Int. J. Syst. Evol. Microbiol. **54:**969–973.
50. **Zhao, H., D. Yang, C. R. Woese, and M. P. Bryant.** 1993. Assignment of fatty acid-beta-oxidizing syntrophic bacteria to *Syntrophomonadaceae* fam. nov. on the basis of 16S rRNA sequence analysis. Int. J. Syst. Bacteriol. **43:**278–286.
51. **Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans van Vliet, J. A. G. M. de Visser, and W. M. de Vos.** 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microb. Ecol. Health Dis. **13:**129–134.