MINIREVIEW

Ecophysiology of syntrophic communities that degrade saturated and unsaturated long-chain fatty acids

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Abstract

Syntrophic relationships are the key for biodegradation in methanogenic environments. We review the ecological and physiological features of syntrophic communities involved in the degradation of saturated and unsaturated long-chain fatty acids (LCFA), as well as their potential application to convert lipids/fats containing waste to biogas. Presently, about 14 species have been described with the ability to grow on fatty acids in syntrophy with methanogens, all belonging to the families *Syntrophomonadaceae* and *Syntrophaceae*. The principle pathway of LCFA degradation is through β-oxidation, but the initial steps in the conversion of unsaturated LCFA are unclear. Communities enriched on unsaturated LCFA also degrade saturated LCFA, but the opposite generally is not the case. For efficient methane formation, the physical and inhibitory effects of LCFA on methanogenesis need to be considered. LCFA adsorbs strongly to biomass, which causes encapsulation of active syntrophic communities and hampers diffusion of substrate and products in and out of the biomass. Quantification of archaea by real-time PCR analysis suggests that potential LCFA inhibitory effect towards methanogens might be reversible. Rather, the conversion of adsorbed LCFA in batch assays was shown to result in a significant increase of archaeal cell numbers in anaerobic sludge samples.

Introduction

Lipids, mainly in the form of neutral fats, are commonly present in domestic sewage and industrial effluents, such as the ones from food-processing industries, wool-scouring facilities and edible oil-processing units. In domestic wastewaters lipids generally represent 20–25% of the total organic matter, with concentrations ranging from 40 to 100 mg L^{-1} (Quémeneur & Marty, 1994). Lipid content of industrial wastewaters is variable and highly dependent on the industrial process. Becker *et al.* (1999) reported the presence of 16.8 ± 5.6 g L^{-1} of lipids in industrial effluent from a wool-scouring process. Average lipid concentration of 10.1 ± 16.6 g L^{-1} was measured in wastewaters from an olive oil-processing plant (Beccari *et al.*, 2002). Relatively lower levels of lipids were detected in wastewaters from a sunflower oil mill (0.4–1.7 g L^{-1}) (Saatci *et al.*, 2003) and from a dairy industry (1.5–4.7 g L^{-1}) (Mendes *et al.*, 2006).

Treatment of lipid-rich wastewaters in anaerobic bioreactors may result in the production of large amounts of biogas, as lipids hold a rather high energetic potential; *c.* 1 L methane can be produced from the complete oxidation of 1 g of lipids. Over 90% of the methane potential from lipids, after their hydrolysis to glycerol and long-chain fatty acids (LCFA), is kept in the LCFA. Hydrolysis of lipids is generally regarded as a fast process, and the rate-limiting step is the degradation of LCFA (e.g. Hanaki *et al.*, 1981; Pavlostathis & Giraldogomez, 1991; Broughton *et al.*, 1998; Becker & Markl, 2000; Masse *et al.*, 2003). The application of anaerobic digestion of lipids/LCFA-rich wastewaters was hindered by problems related to LCFA adsorption to the biomass, with consequent sludge flotation and washout (e.g. Rinzema, 1988; Hwu *et al.*, 1998a, b) and inhibitory/toxic effects of LCFA to different groups of microorganisms (e.g. Hanaki *et al.*, 1981; Koster & Cramer, 1987; Rinzema *et al.*, 1994). Hitherto, removal of lipids/LCFA from the wastewater by physicochemical processes (before anaerobic treatment) has been a common practice with the consequent loss of their energetic potential.

Fatty acids are organic molecules composed of a hydrophilic head, a carboxyl group, and a hydrophobic aliphatic

Table 1. Saturated and unsaturated LCFA commonly found in raw materials and wastewaters (shown as % of total LCFA) (adapted from Hwu, 1997)

	LCFA common name (structure*)						
	Saturated LCFA				Unsaturated LCFA		
Raw materials/wastewaters	Laureate (C12:0)	Myristate (C14:0)	Palmitate (C16:0)	Stearate (C18:0)	Palmitoleate (C16:1)	Oleate (C18:1)	Linoleate (C18:2)
Palm oil†		1.4	42.9	4.8	0.7	39.0	10.0
Olive oil†			14.3	2.4	1.4	71.4	5.5
Soybean oil†		1.0	11.0	4.8		21.9	49.0
Cotton seed oil†		1.4	25.7	2.9	1.0	15.2	51.9
Cocoa butter†			26.7	32.9	0.5	33.8	4.3
Whole milk‡	7.0	6.0	21.0	6.0	2.0	39.0	13.0
Chicken fat†		1.4	21.0	4.3	6.7	42.4	20.0
Beef tallow†	1.0	2.6	28.1	20.0	3.8	37.6	2.9
Domestic sewage§		2.2	16.4	8.1	0.9	30.5	29.2
Dairy wastewater¶			27.0	7.0		37.0	13.0

*C*n*:*d*, where *n* is the number of carbon atoms and *d* the number of double bonds.
†Taylor (1965).
‡Hanaki *et al*. (1981).
§Quémeneur & Marty (1994).
¶Kim *et al*. (2004a).

tail. Aliphatic tails can vary in length and degree of saturation. Carboxylic acids containing > 12 carbon atoms are normally defined as LCFA. The absence or presence of double bonds in the fatty-acid aliphatic chain makes them saturated or unsaturated, respectively. In nature, most common unsaturated LCFA are in the *cis* configuration (e.g. *cis*-9-oleic acid, *cis*-9,12-linoleic acid). LCFA containing one or more *cis* double bonds (*cis*-C*n*:*x*, where *n* is the number of carbons and *x* the number of double bonds) have a more limited flexibility than the corresponding saturated molecules (C*n*:0) or even *trans* isomers (*trans*-C*n*:*x*) (Schneider *et al*., 1949; Seelig & Seelig, 1977; Vollhardt, 2007). Unsaturated LCFA *cis* conformation prevents the chain from having a completely extended configuration as occurs in saturated LCFA or *trans* isomers. Differences in conformations limit the ability of fatty acids to be closely packed. Values of area per molecule in a spread LCFA monolayer are different for unsaturated and saturated LCFA; for example, stearic acid (C18:0) has a limiting area of 20 $Å^2$, while elaidic acid (*trans*-9-C18:1), oleic acid (*cis*-9-C18:1) and linoleic acid (*cis*-9,12-C18:2) have limiting areas of *c*. 33, 41 and 48 $Å^2$, respectively (Kanicky & Shah, 2002). Spatial organization of LCFA is also determined by the chain length. Because, as the chain length increases, van der Waals interactions between the chains of adjacent molecules increase, bringing these molecules closer to each other. Solubility of LCFA in water is rather low and decreases with increasing length of their hydrocarbon chain. When a hydrocarbon radical is large, the strength of the carboxyl group is not enough to bring the molecule of acid to the solution. Solubility of palmitic (C16:0) and stearic (C18:0) acids at 20 °C is 0.72 and 0.29 mg LCFA per 100 g of water; shorter chain fatty acids have higher solubility, for example 79.0 mg of caprylic acid (C8:0) can be dissolved in 100 g of water at 30 °C (Eggenberger *et al*., 1949). Unsaturated LCFA, have slightly higher solubilities; for example, linoleic acid (C18:2) solubility in 6.7 °C water is 16 mg LCFA per 100 mL of water (Mabrouk & Dugan, 1961). In aqueous solutions and neutral pH fatty acids are normally in their ionized form.

Saturated and unsaturated LCFA are present in raw materials and wastewaters (Table 1). Although lipid-containing raw materials and wastewaters significantly differ in their LCFA composition, palmitate and oleate are in general the most abundant saturated and unsaturated LCFA, respectively.

In methanogenic environments interspecies hydrogen transfer between microorganisms plays a central role in LCFA degradation. LCFA are degraded by obligate syntrophic communities of proton-reducing acetogenic bacteria, converting LCFA to acetate and hydrogen/formate, acetoclastic methanogenic archaea and hydrogen/formate-consuming methanogenic archaea (Schink, 1997; Schink & Stams, 2006; Vollhardt, 2007; McInerney *et al*., 2008) (Table 2).

In principle, both saturated and unsaturated LCFA are degraded to acetate and hydrogen via β-oxidation. However, while the degradation of saturated-LCFA follows the classic β-oxidation pathway, the degradation of unsaturated LCFA may require a preliminary step of hydrogenation or an alternative degradation pathway (Weng & Jeris, 1976; Roy *et al*., 1986). This review aims to provide a comprehensive overview of the current state of the art with respect to anaerobic microbial LCFA degradation, focusing on the differences between the degradation of saturated and unsaturated LCFA. A thorough understanding of the microbial diversity and function of LCFA-degrading communities in anaerobic

Table 2. Gibbs free energy changes for some of the acetogenic and methanogenic reactions (presumably) involved in syntrophic conversion of different fatty acids

Reactant	Equation	$\Delta G^{0'}$ (kJ per reaction)*	$\Delta G'$ (kJ per reaction)†
Fatty acids oxidation reactions			
Linoleate (C18:2)	$Linoleate^- + 16H_2O \rightarrow 9\ acetate^- + 14H_2 + 8H^+$	+272	−215
Oleate (C18:1)	$Oleate^- + 16H_2O \rightarrow 9\ acetate^- + 15H_2 + 8H^+$	+338	−177
Stearate (C18:0)	$Stearate^- + 16H_2O \rightarrow 9\ acetate^- + 16H_2 + 8H^+$	+404	−139
Palmitate (C16:0)	$Palmitate^- + 14H_2O \rightarrow 8\ acetate^- + 14H_2 + 7H^+$	+353	−124
Butyrate (C4:0)	$Butyrate^- + 2H_2O \rightarrow 2\ acetate^- + 2H_2 + H^+$	+48	−22
Methanogenic reactions			
Hydrogen	$H_2 + 1/4HCO_3^- + 1/4H^+ \rightarrow 1/4CH_4 + 3/4H_2O$	−34	–
Acetate	$Acetate^- + H_2O \rightarrow HCO_3^- + CH_4$	−31	–

Standard Gibbs energies of formation of LCFA (in aqueous solution, pH 7 and 25 °C) were estimated from the structures of the compounds, using a group contribution method described by Mavrovouniotis (1991); standard Gibbs energies of formation of other compounds involved in the reactions were obtained from Thauer *et al*. (1977).

*Gibbs free energies (at 25 °C) calculated at standard conditions (solute concentrations of 1 M and gas partial pressure of 10^5 Pa).

†Gibbs free energies (at 25 °C) for fatty acids concentrations of 1 mM, considering acetate stoichiometric accumulation (9, 8 or 2 mM for linoleate/oleate/stearate, palmitate and butyrate degradation, respectively) and H_2 depletion to a partial pressure of 1 Pa.

reactors is crucial for the development of new approaches enabling the efficient treatment of LCFA-rich wastewaters.

General biochemical features of LCFA degradation

Biochemical mechanisms of anaerobic LCFA degradation have not been extensively studied in strict anaerobes. Experiments with ^{14}C-labelled palmitate (Nuck & Federle, 1996) and oleate (Weng & Jeris, 1976), as well as the formation of acetate from LCFA degradation under mesophilic (Novak & Carlson, 1970; Hanaki *et al*., 1981; Grabowski *et al*., 2005; Sousa *et al*., 2007b) and thermophilic (Angelidaki & Ahring, 1995; Menes *et al*., 2001) anaerobic conditions, indicate the occurrence of β-oxidation.

Thus far, studies in *Escherichia coli* have given the most complete view on lipids/LCFA metabolism (DiRusso *et al*., 1999; Fujita *et al*., 2007). Wild-type *E. coli* is able to grow aerobically on saturated and unsaturated LCFA as a sole carbon and energy source by means of enzymes encoded by the *fad* regulon (DiRusso *et al*., 1999). A schematic representation of the β-oxidation mechanism of even-numbered LCFA in *E. coli* is shown in Fig. 1. Free fatty acids are activated to acyl-CoA thioesters by acyl-CoA synthetase (FACS, encoded by the *fadD* gene), in a reaction that requires one molecule each of ATP and CoA per molecule of free fatty-acid activated; fatty acyl-CoA is further degraded via β-oxidation in *E. coli* (Fig. 1). The first step in the β-oxidation cycle involves the conversion of acyl-CoA to enoyl-CoA, mediated by an acyl-CoA dehydrogenase. The *fadF* gene encodes an acyl-CoA dehydrogenase with substrate specificity for long- and medium-chain acyl-CoA, while the *fadG* gene encodes a short-chain acyl-CoA dehydrogenase; the *fadE* locus encodes the electron-transferring flavoprotein (ETF) required for the long- and short-chain acyl CoA dehydrogenases (DiRusso *et al*., 1999). Fatty-acid β-oxidation continues through the steps of hydration, oxidation and thiolytic cleavage, with the aid of a multienzyme complex encoded by *fadB* and *fadA* genes (DiRusso *et al*., 1999). The β-oxidation pathway acts in a cyclic manner, each cycle resulting in the shortening of the input acyl-CoA by two carbon atoms to give acetyl-CoA. Degradation of polyunsaturated LCFA by *E. coli* requires Δ^3,Δ^2-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase encoded by *fadB* and *fadH*, respectively (You *et al*., 1989; DiRusso *et al*., 1999). Degradation of oleate (C18:1) and linoleate (C18:2) via the isomerase-dependent pathway is shown in Fig. 1. In *E. coli*, the isomerase-dependent pathway is the main mechanism for the degradation of oleate (Ren *et al*., 2004).

Campbell *et al*. (2003) reported growth of *E. coli* on fatty acids under anaerobic conditions, but only when a terminal respiratory electron acceptor, such as nitrate or fumarate, was available. Homologues of the *fadA* and *fadB* genes, required for aerobic fatty-acid utilization, called *yfcY* and *yfcX*, respectively, are involved in anaerobic growth on fatty acids. MS experiments showed further that the anaerobic degradation pathway proceeds by cleavage of the carbon chain between the β and γ carbons as in the classical β-oxidation pathway (Campbell *et al*., 2003).

Microbiology of LCFA degradation

Syntrophic LCFA-degrading bacteria

Anaerobic LCFA-degrading bacteria have not been isolated from methanogenic environments until the mid-1980s. *Syntrophomonas sapovorans* – from Latin meaning 'soap devouring' – was the first bacterium described that degraded

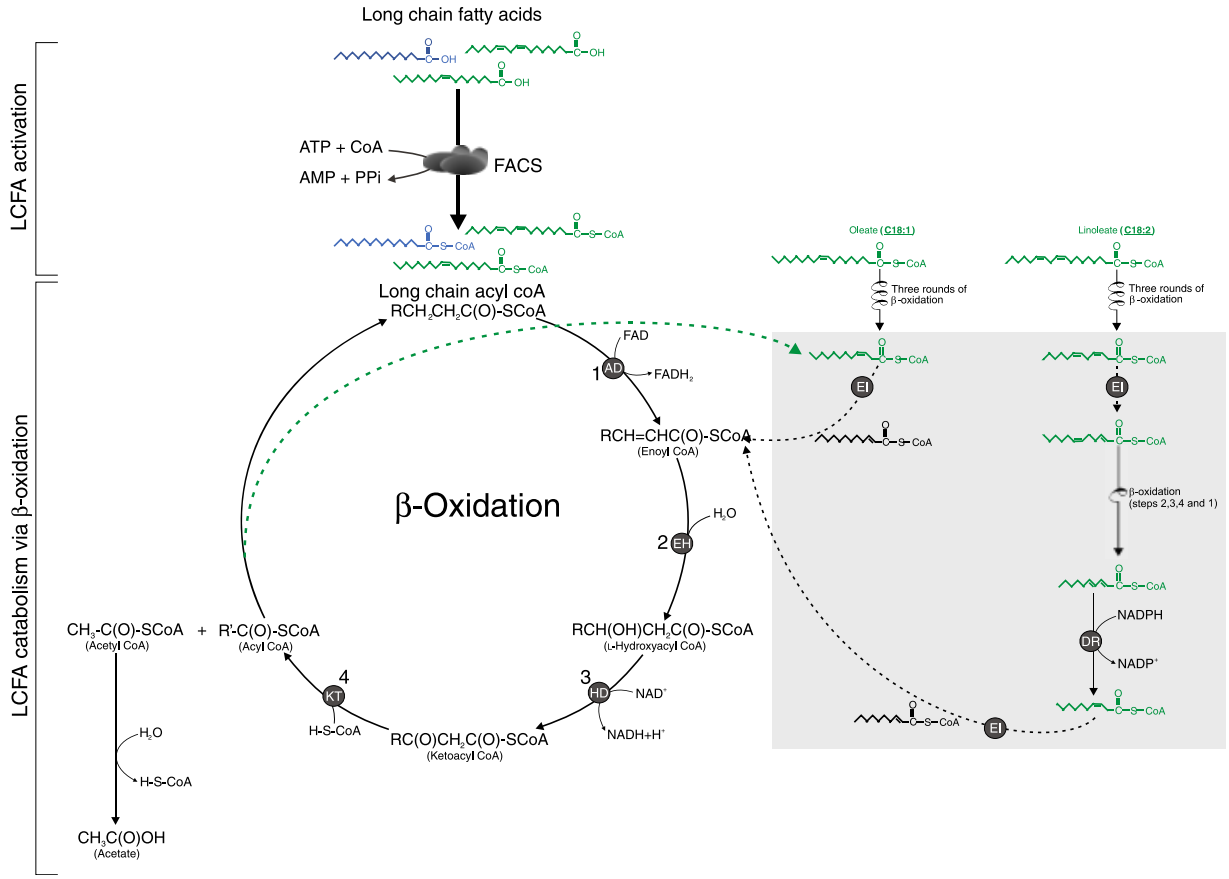

Fig. 1. LCFA catabolism via cyclic β-oxidation in *Escherichia coli* (even-numbered LCFA). On the right square, degradation of unsaturated LCFA via isomerase-dependent pathway is exemplified for oleate (C18:1) and linoleate (C18:2) (fatty acids and intermediates of degradation that are unsaturated are represented in green). Enzymes catalysing each of the reactions of the pathway (and respective encoding genes) are: FACS, acyl-CoA synthetase (*fadD*); AD, acyl-CoA dehydrogenase (*fadF, fadG*) and ETF (*fadE*); EH, enoyl CoA hydratase (*fadB*); HD, L-3 hydroxyacyl dehydrogenase (*fadB*); KT, 3-ketoacyl-CoA thiolase (*fadA*); EI, Δ^3,Δ^2-enoyl-CoA isomerase (*fadB*); DR, 2,4-dienoyl-CoA reductase (*fadH*) (adapted from Yang *et al.*, 1986; DiRusso *et al.*, 1999; Ren *et al.*, 2004).

saturated and unsaturated LCFA in coculture with *Methanospirillum hungatei* (Roy *et al.*, 1986). To date, 14 fatty-acid-degrading syntrophic bacteria have been obtained in pure culture or in coculture with hydrogen-consuming microorganisms (Table 3). They all belong to the families *Syntrophomonadaceae* (McInerney, 1992; Zhao *et al.*, 1993; Wu *et al.*, 2006b; Sousa *et al.*, 2007c) and *Syntrophaceae* (Jackson *et al.*, 1999), within the phyla *Firmicutes* and *Deltaproteobacteria*, respectively. Syntrophic bacteria function together with hydrogenotrophic archaea or hydrogen-consuming sulphate-reducing bacteria during fatty-acid degradation. LCFA (defined as fatty acids with >12 carbon atoms) are utilized by *S. sapovorans* (Roy *et al.*, 1986), *Syntrophomonas saponavida* (Lorowitz *et al.*, 1989; Wu *et al.*, 2007), *Syntrophomonas curvata* (Zhang *et al.*, 2004), *Syntrophomonas zehnderi* (Sousa *et al.*, 2007c), *Syntrophomonas palmitatica* (Hatamoto *et al.*, 2007a), *Thermosyntrophica lipolytica* (Svetlitshnyi *et al.*, 1996) and *Syntrophus aciditrophicus* (Jackson *et al.*, 1999). Among these microorganisms, the capability of utilizing mono- and/or polyunsaturated LCFA with >12 carbon atoms is restricted to *S. sapovorans*, *S. curvata*, *S. zehnderi* and *T. lipolytica*. Thus far no studies have been performed on the biochemical mechanisms of saturated and unsaturated LCFA degradation by these syntrophic microorganisms.

Ecology of methanogenic LCFA-degrading communities

Fatty-acid β-oxidizing bacteria are extremely fastidious microorganisms (Sobieraj & Boone, 2006) and sometimes difficult to grow in pure culture or coculture with a hydrogen-consuming partner. Nevertheless, new insights with respect to the identity, physiology and ecology of LCFA-degrading bacteria have been obtained with culture-independent molecular techniques. Figure 2 shows an

Table 3. Fatty acid degrading syntrophic bacteria

	Substrate utilization in coculture with a syntrophic partner											
	Butyrate (C4:0)	Caproate (C6:0)	Caprylate (C8:0)	Caprate (C10:0)	Laurate (C12:0)	Myristate (C14:0)	Palmitate (C16:0)	Stearate (C18:0)	Oleate (C18:1)	Linoleate (C18:2)	Syntrophic partner used	References
Syntrophomonas bryantii	+	+	+	+	–	–	–	–	ND	ND	*Methanospirillum hungatei* *Desulfovibrio* sp. E70	Stieb & Schink (1985), Zhao *et al*. (1990), Wu *et al*. (2006a)
Syntrophomonas cellicola	+	+	+	–	–	–	ND	ND	ND	ND	*Methanobacterium formicicum* *Desulfovibrio* sp. G11	Wu *et al*. (2006c)
Syntrophomonas curvata	+	+	+	+	+	+	+	+	+	–	*Methanobacterium formicicum*	Zhang *et al*. (2004, 2005)
Syntrophomonas erecta erecta	+	+	+	–	–	–	–	–	–	–	*Methanospirillum hungatei*	Zhang *et al*. (2005), Wu *et al*. (2006b)
Syntrophomonas erecta sporosyntropha	+	+	+	–	–	–	–	–	–	–	*Methanobacterium formicicum*	Wu *et al*. (2006b)
Syntrophomonas palmitatica	+	+	+	+	+	+	+	+	–	–	*Methanobacterium formicicum*	Hatamoto *et al*. (2007a)
Syntrophomonas saponavida	+	+	+	+	+	+	+	+	–	–	*Methanospirillum hungatei* *Desulfovibrio* sp. G11	Lorowitz *et al*. (1989), Wu *et al*. (2007)
Syntrophomonas sapovorans	+	+	+	+	+	+	+	+	+	+	*Methanospirillum hungatei*	Roy *et al*. (1986), Zhang *et al*. (2005)
Syntrophomonas wolfei methylbutyratica	+	+	+	–	–	–	–	–	ND	ND	*Methanobacterium formicicum*	Wu *et al*. (2007)
Syntrophomonas wolfei wolfei	+	+	+	–	–	–	–	–	–	–	*Methanospirillum hungatei* *Desulfovibrio* sp. G11	McInerney *et al*. (1979, 1981), Zhang *et al*. (2005)
Syntrophomonas zehnderi	+	+	+	+	+	+	+	+	+	±	*Methanobacterium formicicum*	Sousa *et al*. (2007c)
Syntrophus aciditrophicus	+	+	+	ND	ND	ND	+	+	ND	ND	*Methanospirillum hungatei* *Desulfovibrio* sp. G11	Jackson *et al*. (1999)
Syntrophothermus lipocalidus	+	+	+	+	–	–	–	–	–	–	*Methanobacterium thermoautotrophicum*	Sekiguchi *et al*. (2000)
Thermosysntropha lipolytica	+	+	+	+	+	+	+	+	+	+	*Methanobacterium* strain JW/VS-M29	Svetlitshnyi *et al*. (1996)

ND, not determined or not reported. Substrate utilisation by the different strains in coculture with the syntrophic partner: +, utilized; ±, poorly utilized; –, not utilized.

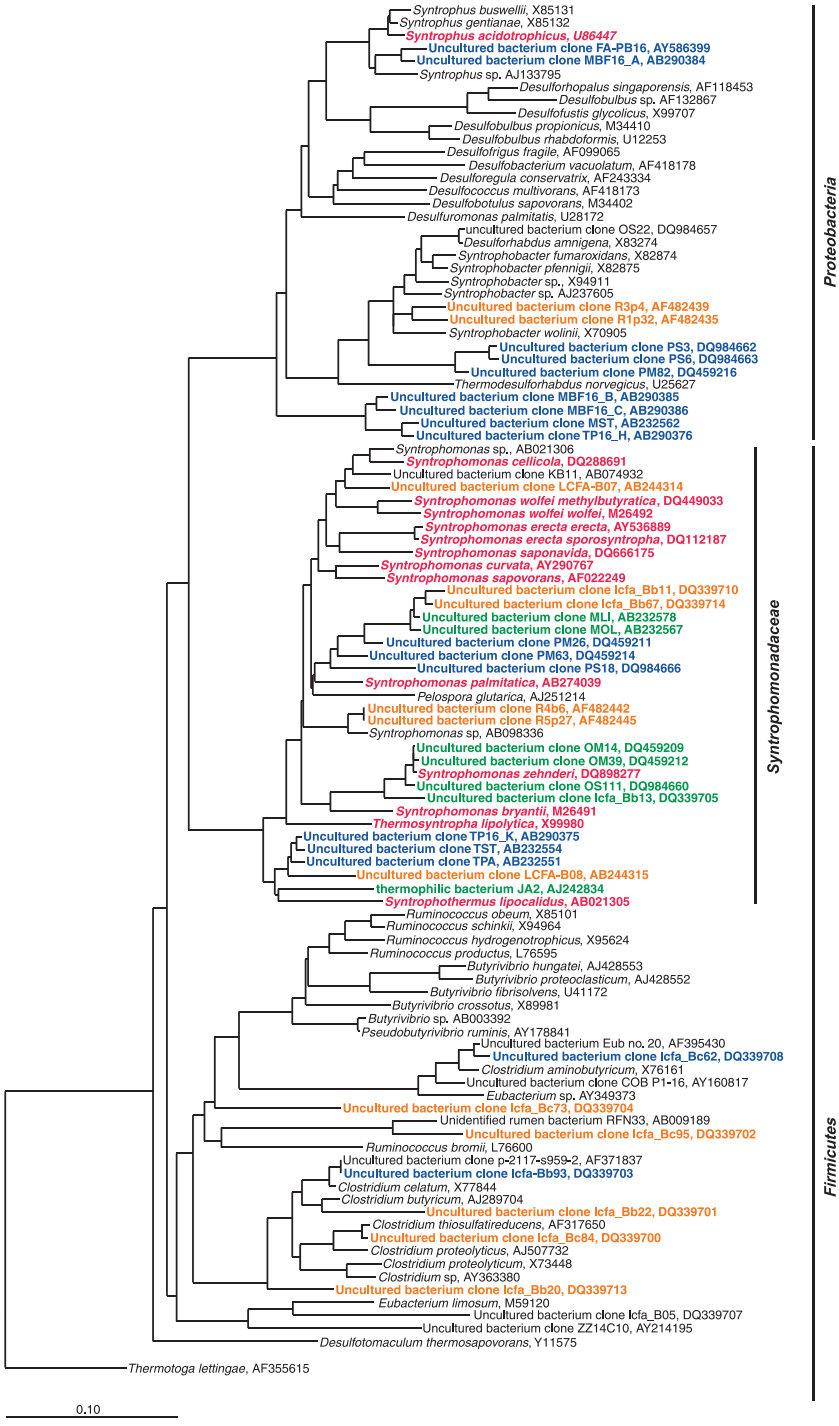

Fig. 2. Phylogenetic tree of 16S rRNA gene sequences retrieved from different LCFA-degrading microbial communities. The following colour codes were used: red, described fatty-acid degrading acetogenic bacteria; green, clones retrieved from microbial communities degrading unsaturated LCFA; blue, clones retrieved from microbial communities degrading saturated LCFA; orange, clones retrieved from microbial communities degrading LCFA mixtures. 16S rRNA gene sequences were obtained from the NCBI nucleotide database, and respective GenBank accession numbers are shown as reference. Tree was calculated using the ARB software package (Ludwig *et al*., 2004) and applying the neighbour-joining method (Saitou & Nei, 1987). *Thermotoga lettingae* (AF355615) was used as outgroup.

overview of the phylogenetic affiliation (based on the16S rRNA gene) of several microorganisms present in LCFA-degrading communities, including those observed in enrichment cultures or bioreactors' sludge.

Clone libraries derived from LCFA-degrading communities point towards the importance of *Firmicutes* and *Proteobacteria* members during the degradation of these compounds (Menes *et al*., 2001; Grabowski *et al*., 2005; Hatamoto *et al*., 2007b, c; Sousa *et al*., 2007a, b, 2009). A significant number of *Syntrophomonadaceae*-related microorganisms, clustering together with described syntrophic LCFA-degrading bacteria, were detected in microbial

communities degrading both unsaturated and saturated LCFA. Shigematsu *et al.* (2006) operated an anaerobic chemostat fed with a mixture of oleate (C18:1) and palmitate (C16:0). The biomass that was enriched consisted of bacteria affiliated with the *Syntrophomonadaceae* family. *Syntrophomonas*-related microorganisms were present in anaerobic bioreactor sludges submitted to continuous oleate (C18:1)- and palmitate (C16:0)-feeding followed by batch degradation of the accumulated LCFA (Sousa *et al.*, 2007a). Microorganisms from this genus were further identified as key players in oleate and palmitate enrichment cultures (Sousa *et al.*, 2007b, 2009). Even when incubated with sulphate, oleate- and palmitate-degrading cultures showed a predominance of syntrophic LCFA-degrading bacteria and no sulphate-reducing LCFA-degrading bacteria were detected (Sousa *et al.*, 2009). Hatamoto *et al.* (2007b) studied the phylogeny of microorganisms present in thermophilic and mesophilic palmitate (C16:0), stearate (C18:0), oleate (C18:1) and linoleate (C18:2) enrichment cultures. Thermophilic palmitate and stearate enrichment cultures showed a predominance of *Syntrophothermus*-related microorganisms, while microorganisms closely related to *Tepidanaerobacter syntrophicus* were predominant in the thermophilic oleate enrichment culture. A bacterial strain (strain TOL) with high rRNA gene resemblance with *T. syntrophicus* was isolated, but was not able to degrade LCFA. Bacteria affiliated with the genus *Syntrophomonas* were predominant in mesophilic enrichment cultures growing on palmitate, oleate and linoleate, with *S. curvata* and *S. sapovorans* as the closest cultivated species. Members of the class *Deltaproteobacteria*, though distantly related to cultivated species, were the most abundant bacteria detected in the stearate mesophilic enrichment culture. In a latter study, Hatamoto *et al.* (2007c) detected a significant abundance of *Syntrophaceae* members in 16S rRNA gene clone library generated from ^{13}C-labeled RNA fractions of a mesophilic palmitate (C16:0) enrichment. *Syntrophus aciditrophicus* is the only deltaproteobacterium belonging to the *Syntrophaceae* family with the ability to degrade LCFA. Grabowski *et al.* (2005) characterized stearate (C18:0) and heptanoate (C17:0) methanogenic cultures enriched from a low-temperature biodegraded oil reservoir. These authors also found *Syntrophus*-related species as predominant members of the LCFA enrichment cultures.

Factually, even though LCFA-degrading *Syntrophomonadaceae* members are commonly found in clone libraries from bioreactors treating effluents with high LCFA content, numbers of this family in such bioreactors are apparently low (Hansen *et al.*, 1999; Menes & Travers, 2006). Hansen *et al.* (1999) described the development and application of new radioactively labelled oligonucleotide probes to characterize the phylogenetic groups of mesophilic members of the family *Syntrophomonadaceae*. They found that 0.2–1% of the 16S rRNA gene present in a full-scale biogas plant treating a mixture of swine-cattle manure and industrial organic waste belonged to members of the *Syntrophomonadaceae*, of which the majority was accounted for by the genus *Syntrophomonas*. More recently, Menes & Travers (2006) used FISH to quantify *Syntrophomonadaceae* members in anaerobic sludge from a reactor treating edible tallow refinery wastewater. In this case, the number of FISH-positives belonging to the *Syntrophomonadaceae* family represented 3% of EUB338 probe-positive cells. Other potentially LCFA-degrading groups, such as the members of the *Syntrophaceae* family mentioned above, were never quantified in LCFA-degrading communities. The detection of only low numbers of *Syntrophomonadaceae*, while the LCFA-degrading communities present in LCFA-degrading bioreactors or enrichments are rather diverse, have opened the discussion on the LCFA-degrading potential of bacterial families other than *Syntrophomonadaceae* and *Syntrophaceae* (Shigematsu *et al.*, 2006; Hatamoto *et al.*, 2007b, c; Sousa *et al.*, 2007a). Hatamoto *et al.* (2007c) used RNA-based stable isotope probing (SIP) for linking microbial function and structure during the degradation of palmitate (C16:0). These authors incubated different mesophilic and thermophilic anaerobic sludges with ^{13}C-labelled palmitate ([1,2,3,4-$^{13}C_4$] palmitic acid potassium salt), after which they constructed clone libraries from ^{13}C-labelled RNA fractions. *Syntrophomonadaceae*- and *Syntrophaceae*-related microorganisms were detected in these libraries, but the majority of clones found in the heavy rRNA fraction were close relatives of different species belonging to genera not usually associated to LCFA degradation, namely *Clostridium*-, *Thermotoga*-, *Coprothermobacter*- and *Anaerobaculum*-related species. However, the involvement of these species in palmitate degradation was not clear. *Clostridium*-related bacteria have been detected in other microbial communities that degrade LCFA (Fig. 2). In a previous study we found that about one-third of the predominant denaturing gradient gel electrophoresis bands present in LCFA-degrading sludge fingerprints corresponded to *Clostridiaceae* members (Sousa *et al.*, 2007a). Those results led us to test the degradation of LCFA by *Clostridium* species. Several species, namely *Clostridium butyricum*, *Clostridium magnum*, *Clostridium beijerinckii*, *Clostridium amygdalinum*, *Clostridium methoxybenzovorans*, *Clostridium ultunense* and *Clostridium thermocellum*, were tested for the capability to degrade medium- and long-chain fatty acids in coculture with hydrogenotrophic methanogens, but degradation was never observed (unpublished data). Many *Thermotoga* species have been isolated from oil reservoirs or oil wells, for example, *Thermotoga elfii*, *Thermotoga hypogeal*, *Thermotoga petrophila* and *Thermotoga naphtophila*. This could be an indication that these microorganisms can endure high concentrations of LCFA, but no data are available that show that they can use these compounds. We

tested degradation of LCFA by *T. maritima* in coculture with a hydrogenotrophic methanogen, but we did not find degradation of the LCFA (unpublished data). Menes *et al.* (2001) characterized a thermophilic oleate-degrading enrichment using amplified ribosomal DNA restriction analysis (ARDRA). They found three predominant ARDRA patterns corresponding to bacteria related to *Coprothermobacter* and *Anaerobaculum* species, together with a syntrophic bacterium most closely related to *Syntrophothermus lipocalidus*. The nonsyntrophic bacteria were both isolated from the oleate enrichment culture: strain J4 (related to *Coprothermobacter proteolyticus*) and strain NGA [later classified as *Anaerobaculum mobile* (Menes & Muxi, 2002)]. Oleate and smaller fatty acids, such as butyrate, were not degraded by these strains in coculture with *Methanthermobacter thermoautotrophicus*. Their persistence in the enrichment cultures was justified by their ability to use proteinaceous substrates, resulting from decaying cells. So, it is not clear yet if other microorganisms than members of *Syntrophomonadaceae* or *Syntrophaceae* families are able to degrade LCFA. Most likely, the presence of nonsyntrophic bacteria in LCFA-degrading communities is related to the conversion of side compounds formed during LCFA degradation. This may enable these bacteria to outnumber the slowly growing syntrophic LCFA degraders. *Syntrophomonadaceae* and *Syntrophaceae* members were not predominantly detected in the ^{13}C-labelled RNA fraction (Hatamoto *et al.*, 2007c). However RNA-SIP results might be ambiguous due to low growth rate of some microorganisms and cross-feeding during the enrichment phase, as well as due to biases introduced during PCR amplification (Radajewski *et al.*, 2003). In the work from Hatamoto *et al.* (2007c) short incubation periods were used to prevent cross-feeding, but this might have also affected the detection of slow-growing microorganisms, as is the case of syntrophic LCFA degraders.

The saturated/unsaturated-LCFA specificity of different microorganisms is also not yet clear. Differences in microbial communities developed in the presence of unsaturated and saturated LCFA were previously described using oleate (C18:1) and palmitate (C16:0) as substrates (Sousa *et al.*, 2009). Highly specialized palmitate enrichment cultures could degrade saturated LCFA, with four to 18 carbon atoms, but could not degrade oleate, while oleate enrichment cultures were more versatile (Sousa *et al.*, 2007b). A close relative of *S. zehnderi* was identified as the potential key population in the degradation on unsaturated LCFA in those oleate enrichment cultures. Considering the phylogeny depicted in Fig. 2, one can see that several 16S rRNA gene sequences retrieved from unsaturated-LCFA degrading communities cluster within the *Syntrophomonadaceae* family. On the other hand, 16S rRNA gene sequences retrieved from saturated-LCFA-degrading communities form a compact cluster deeply branching within the *Deltaproteobacteria*.

Interspecies metabolites transfer in LCFA degradation

In methanogenic environments syntrophic LCFA degradation is dependent on the activity of hydrogenotrophic microorganisms, which maintain hydrogen concentration at low levels (Sousa *et al.*, 2007b). The possible involvement of formate as an alternative electron carrier during LCFA degradation has not been studied. Hydrogenotrophic archaea or sulphate-reducing bacteria used as hydrogen scavengers in syntrophic LCFA cocultures (Table 2) can also utilize formate, which makes it difficult to quantify the extent of hydrogen and/or formate removal. Oxidation of butyrate by *Syntrophomonas bryantii* (formerly *Syntrophosphora bryantii*) require syntrophic partners that are able to use both hydrogen and formate, indicating that both carriers are involved in interspecies electron transfer (Dong *et al.*, 1994; Dong & Stams, 1995a, b). Nonetheless, hydrogen was considered as the main electron carrier because hydrogenase activities in *S. bryantii* cocultures substantially exceed formate dehydrogenase activities. During degradation of C4- to C18-fatty acids by LCFA enrichment cultures, formate was not detected in the medium (Sousa *et al.*, 2007b), but in that study the detecting limit for formate was 5 mM. It cannot be excluded that lower concentrations are present in those cultures. The role of formate during LCFA degradation remains unclear and needs further investigation. Acetoclastic archaea have an important role in the overall efficient conversion of LCFA to methane, because methane derived from acetate accounts for about 70% of the total theoretical methane potential of LCFA. Energetics of syntrophic degradation of LCFA does not rely on the activity of acetate-consuming microorganisms (Table 2), but at low acetate concentration higher hydrogen and formate levels can be tolerated (Table 3). Some authors described toxic and inhibitory effects of LCFA on acetoclastic methanogens (Hanaki *et al.*, 1981; Koster & Cramer, 1987; Angelidaki & Ahring, 1995; Lalman, 2000; Lalman & Bagley, 2001). Inhibition of hydrogenotrophic methanogens by LCFA was also reported (Demeyer & Henderic, 1967; Hanaki *et al.*, 1981; Lalman, 2000; Lalman & Bagley, 2001), although much less severely than that observed with acetoclastic methanogens. These statements on LCFA toxicity/inhibition were derived from the interpretation of batch experiments, in which there was a decrease of the methanogenic activity. Pereira *et al.* (2005) explained the decrease of methanogenic activity, after cell contact with LCFA, as being a temporary effect resulting mainly from mass transfer limitations. Also, when LCFA are adsorbed to biomass they can be efficiently converted to methane and, after this conversion, specific methanogenic activity increases (Pereira *et al.*, 2004). Quantification of archaea by 16S rRNA gene-targeted real-time PCR in sludge samples collected from continuously LCFA-

fed bioreactors – containing high amounts of adsorbed LCFA – and the same samples after batch degradation of the adsorbed substrate do not support the theory of methanogenic inhibition by these compounds. An increase in the relative abundance of archaea was observed after batch depletion of the accumulated substrate; sludge samples collected from continuous reactors fed with oleate and palmitate showed a relative archaea abundance of $48 \pm 9.0\%$ and $35.4 \pm 14.3\%$ of the total microbial community, respectively. After batch degradation of the adsorbed substrate, these values were increased to $85 \pm 29\%$ and $75 \pm 14\%$, respectively (Sousa *et al.*, 2007a). These results suggest a possible endurance of methanogens after contact with LCFA. Cell death or damage of methanogens after contact with LCFA have never been directly observed. LCFA toxicity/inhibition towards methanogens needs to be further assessed as it can harm efficient hydrogen and acetate uptake, disabling an efficient conversion of these substrates to methane.

Analysis of the diversity of methanogenic archaea in upflow bioreactors fed with oleate or palmitate evidenced a predominance of hydrogenotrophs belonging to the genera *Methanobacterium* (Pereira *et al.*, 2002b; Sousa *et al.*, 2007a). In these studies, acetoclastic archaea comprised of mainly *Methanosaeta* and *Methanosarcina* species. Also, Shigematsu *et al.* (2006) analysed the archaeal composition of anaerobic communities present in chemostats fed with a mixture of oleate (C18:1) and palmitate (C16:0), and found predominantly the hydrogenotrophic genus *Methanospirillum* and the acetoclastic genera *Methanosaeta* and *Methanosarcina*. Competition of methanogens with hydrogen- and acetate-consuming sulphate-reducing bacteria was shown to occur in anaerobic enrichment cultures degrading oleate and palmitate (Sousa *et al.*, 2009). *Desulfovibrio*-, *Desulfomicrobium*- and *Desulforhabdus*-related species could overgrow methanogens present in oleate- and palmitate-degrading cultures after subculturing in the presence of 10 mM of sulphate (Sousa *et al.*, 2009). If this competition outcome is a result of differences in kinetics or on cell resistance to the presence of LCFA is not clear at present.

Differences in saturated and unsaturated LCFA degradation

Early studies suggested that the degradation of unsaturated LCFA requires complete chain saturation before β-oxidation (Novak & Carlson, 1970; Weng & Jeris, 1976). Unsaturated LCFA such as linoleate (C18:2) and oleate (C18:1) would be metabolized through a hydrogenation step yielding stearate (C18:0), which then enters the β-oxidation cycle. Assuming that unsaturated LCFA are degraded by these two sequential steps – hydrogenation and β-oxidation – it is not clear if these steps are performed by one or more microorganisms. Some syntrophic LCFA-degrading bacteria can use unsaturated LCFA as sole carbon and energy source, but some fermentative bacteria are able to hydrogenate unsaturated LCFA (Mackie *et al.*, 1991). Biohydrogenation of unsaturated LCFA by rumen bacteria is best studied (e.g. Polan *et al.*, 1964; van de Vossenberg & Joblin, 2003; Maia *et al.*, 2007; Paillard *et al.*, 2007; Jenkins *et al.*, 2008). Recently, Devillard *et al.* (2007) also reported the metabolism of linoleate by human gut bacteria. Conversion of C18 unsaturated LCFA to stearate involves positional and geometric isomerization of the double bonds coupled with one or more hydrogenations. Linoleate (*cis*-9,*cis*-12-C18:2) is metabolized via conjugated linoleate (mainly *cis*-9,*trans*-11-C18:2) to vaccenate (*trans*-11-C18:1) (Devillard *et al.*, 2007) (Fig. 3a). Hydrogenation of oleate (*cis*-9-C18:1) to stearate involves a *trans*-isomerization to vaccenate (*trans*-11-C18:1) (Fig. 3b). Biohydrogenation of linoleate (C18:2) in the rumen is thought to be carried out mainly by strains belonging to the *Butyrivibrio* genus (Polan *et al.*, 1964; van de Vossenberg & Joblin, 2003; Wallace *et al.*, 2006; Moon *et al.*, 2008). Most of the microorganisms from this group convert linoleate (*cis*-9,*cis*-12-C18:2) to vaccenate (*trans*-11-C18:1), but further reduction to stearate was only observed in cultures of *Butyrivibrio hungatei* strain Su6 (van de Vossenberg & Joblin, 2003) and *Butyrivibrio proteoclasticus* (Wallace *et al.*, 2006; Moon *et al.*, 2008). Stearate formation from unsaturated C18 acids was also observed in cultures of *Fusocillus babrahamensis* and an unidentified *Fusocillus* sp. (Kemp *et al.*, 1975, 1984). However, these *Fusocillus* isolates have been lost and no representatives of this genus are available (Wallace *et al.*, 2006). Other species able to convert linoleate to vaccenate include the ruminal spirochaete *Borrelia* sp. $B_2$5 (Sachan & Davis, 1969) and *Roseburia* spp. isolated from human faeces (Devillard *et al.*, 2007). Figure 3c depicts an alternative LCFA saturation pathway consisting in the hydration of the fatty-acid molecule. This is a mechanism that occurs in the rumen and human gut and results in the formation of several hydroxyl acids (Hudson *et al.*, 2000; Devillard *et al.*, 2007). Microorganisms able to perform this step are rather diverse, including species from *Acetobacterium*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Faecalibacterium*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Propionibacterium*, *Roseburia*, *Selenomonas* and *Streptococcus* genera (Hudson *et al.*, 2000; Devillard *et al.*, 2007).

Some studies suggest that β-oxidation might occur before fatty-acid chain saturation (Roy *et al.*, 1986; Canovas-Diaz *et al.*, 1991). Oleate (C18:1) has been detected as an intermediary of linoleate (C18:2) degradation (Lalman & Bagley, 2000), but stearate (C18:0) was never observed during linoleate (C18:2) or oleate (C18:1) degradation (Canovas-Diaz *et al.*, 1991; Lalman & Bagley, 2000, 2001). Notwithstanding, other saturated LCFA, such as palmitate (C16:0) and myristate (C14:0), were rapidly produced during the degradation of linoleate (C18:2) and oleate

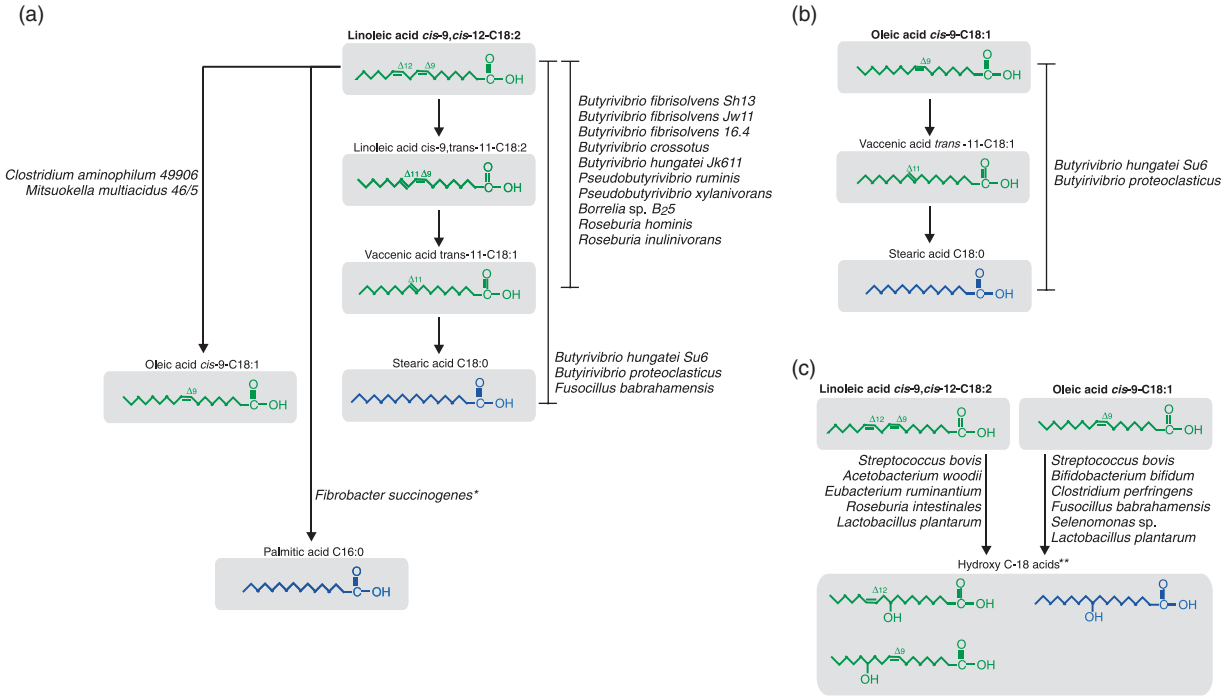

Fig. 3. Simplified pathways of (a) linoleate and (b) oleate biohydrogenation by ruminal microorganisms (van de Vossenberg & Joblin, 2003; Maia *et al.*, 2007). Alternative (c) pathway of linoleate and oleate hydration to hydroxyl C-18 compounds (Hudson *et al.*, 2000; Devillard *et al.*, 2007). Some of the microorganisms able to perform the different conversions are indicated in the figure. **Fibrobacter succinogenes*; product appeared to be palmitic acid though that result was not confirmed by MS. **10-Hydroxy-*cis*-12-octadecenoic, 13-hydroxy-9-octadecenoic acid and 10-hydroxystearic acids are common intermediaries resulting from unsaturated linoleic and oleic acid hydration, but other hydroxyl C-18 compounds can be formed. References: *Clostridium aminophilum* (Paster *et al.*, 1993; Maia *et al.*, 2007), *Mitsuokella multacidus* 46/5 (Maia *et al.*, 2007), *Butyrivibrio fibrisolvens* (Polan *et al.*, 1964; Kepler *et al.*, 1966; Fukuda *et al.*, 2005), *Butyrivibrio hungatei* JK611 (Kopecny *et al.*, 2003; Maia *et al.*, 2007), *B. hungatei* Su6 (van de Vossenberg & Joblin, 2003), *Butyrivibrio proteoclasticus* (Wallace *et al.*, 2006; Moon *et al.*, 2008), *Borrelia* sp. $B_2$5 (Sachan & Davis, 1969), *Pseudobutyrivibrio ruminis* (van Gylswyk *et al.*, 1996), *Acetobacterium woodii* (Giesel-Bühler *et al.*, 1987), *Streptococcus bovis* (Hudson *et al.*, 1998, 2000), *Bifidobacterium bifidum* (Thomas, 1972), *Roseburia intestinales* (Devillard *et al.*, 2007), *Clostridium perfringens* (Thomas, 1972), *Fusocillus babrahamensis* (Kemp *et al.*, 1975), *Selenomonas* sp. (Hudson *et al.*, 1995), *Lactobacillus plantarum* (Yamada *et al.*, 1996; Hudson *et al.*, 2000).

(C18:1) (Canovas-Diaz *et al.*, 1991; Lalman & Bagley, 2000, 2001; Pereira *et al.*, 2002a).

If we considered a low hydrogen pressure for both hydrogenation and β-oxidation of LCFA are energetically favourable. For example, considering a hydrogen partial pressure of 1 Pa, the Gibbs free energy for hydrogenation and β-oxidation reactions is as follows (values calculated with data from Thauer *et al.*, 1977; Mavrovouniotis, 1991):

Hydrogenation:

$$\text{Oleate}^- \text{ (C18:1)} + H_2 \rightarrow \text{stearate}^- \text{ (C18:0)}, \quad \Delta G' = -66\,\text{kJ}$$

β-*Oxidation*:

$$\text{Oleate}^- \text{ (C18:1)} + 2H_2O \rightarrow \text{palmitoleate}^- \text{ (C16:1)} + \text{acetate}^- + 2H_2 + H^+, \quad \Delta G' = 51\,\text{kJ}$$

$$\text{Stearate}^- \text{ (C18:0)} + 2H_2O \rightarrow \text{palmitate}^- \text{ (C16:0)} + \text{acetate}^- + 2H_2 + H^+, \quad \Delta G' = 51\,\text{kJ}$$

Hydrogenation+β-oxidation:

$$\text{Oleate}^- \text{ (C18:1)} + 2H_2O \rightarrow \text{palmitate}^- + \text{acetate}^- + \text{H2} + H^+, \quad \Delta G' = -15\,\text{kJ}$$

Thus far, experimental data are lacking to unequivocally rule out one or the other pathway for the degradation of unsaturated LCFA in anaerobic sludge.

Optimization of LCFA conversion to methane in anaerobic bioreactors

Treating lipids/LCFA-rich wastewaters in anaerobic bioreactors yields high amounts of methane-rich biogas. The two major problems in the anaerobic treatment of fats and lipids containing wastewaters are (1) sludge flotation and biomass washout due to adsorption of lipids/LCFA onto the biomass (e.g. Rinzema, 1988; Hwu *et al.*, 1998a, b) and (2) microbial activity inhibition by LCFA (e.g. Hanaki *et al.*, 1981; Koster & Cramer, 1987; Rinzema *et al.*, 1994). Identification and study of these drawbacks led to the development of new

solutions, and latest results in the anaerobic digestion of effluents containing high amounts of lipids/LCFA are promising (Kim *et al.*, 2004b; Pereira *et al.*, 2004; Haridas *et al.*, 2005; Cavaleiro *et al.*, 2008). Kim *et al.* (2004b) reported an enhanced LCFA conversion to methane in a two-phase bioreactor, composed of a continuous stirred tank reactor for acidogenesis and an upflow anaerobic sludge blanket reactor for methanogenesis. Haridas *et al.* (2005) described the application of a novel reactor, the buoyant filter bioreactor, for the treatment of a fat-rich dairy wastewater, and obtained an almost complete substrate conversion to methane for a period of over 400 days of operation. LCFA adsorption during continuous oleate feeding in anaerobic bioreactors was confirmed by microscopic observation of Sudan Black B stained samples (Pereira *et al.*, 2004). LCFA adsorption onto the sludge was previously observed by Hwu *et al.* (1998b) and it was considered as a necessary condition for LCFA degradation. In the study by Pereira *et al.* (2004), 38 sludge samples, collected from oleate-fed bioreactors and containing different amounts of adsorbed substrate, were incubated in batch assays in order to assess the optimal conditions for LCFA conversion to methane. LCFA accumulation in bioreactors, occurring during long-term operation at high LCFA-loading rates, can cause limitations in the transport of substrate to the biomass with consequent decrease on the removal efficiency. A value of about 1000 mg COD-LCFA g^{-1} biomass was obtained for the optimal specific adsorbed-LCFA content that allowed the maximal degradation rate. Measurement of specific hydrogenotrophic and acetoclastic activities of anaerobic sludges, before and after degradation of the adsorbed LCFA, showed a significant increase in specific methanogenic activities after batch degradation of the adsorbed-LCFA (Pereira *et al.*, 2004). Sequential reactor operation, in which a first step of LCFA adsorption is followed by batch degradation of the biomass-associated substrate, was found to be a possible solution for the treatment of this type of wastewaters (Pereira *et al.*, 2004; Cavaleiro *et al.*, 2008). Recently, Cavaleiro *et al.* (2009) showed that continuous treatment of LCFA can also be achieved after a start up with stepwise increased feeding rate. Based on these findings, a novel high rate anaerobic reactor specifically designed for the treatment of lipids/LCFA wastewaters has been developed (Alves *et al.*, 2007). Because conventional primary biomass retention techniques, such as granulation or biomass fixation, cannot be applied for the treatment of LCFA-rich wastewaters, an alternative system for sludge retention was developed. In this reactor, sludge flotation is used as the primary form of biomass retention. Therefore, the name selected for this system – Inverted Anaerobic Sludge Bed reactor. A secondary system for the retention of settling biomass is included in the reactor. In brief, LCFA present in the influent stream adsorb to biomass, which makes it float; after adsorbed-LCFA degradation, biomass density increases and biomass settles in the bottom of the reactor. Adsorption of LCFA to sludge is stimulated by promoting a close contact between the influent and a recycled stream of settled sludge.

Concluding remarks

For many years, most of the interest in anaerobic biodegradation of LCFA was focused on the process and technology developments, while detailed microbiological studies were lacking. Thus far, anaerobic bacterial isolates that grow on LCFA in syntrophy with methanogens belong to the the *Firmicutes* (*Syntrophomonadacaea*) or the *Deltaproteobacteria* (*Syntrophaceae*). β-Oxidation is the principle pathway of LCFA degradation, but detailed biochemical studies are lacking. In particular, the initial steps in the conversion of unsaturated LCFA need to be resolved. Currently, the genomes of *Syntrophomonas wolfei* (saturated short/medium-chain fatty-acid degrader) and *S. zehnderi* (saturated and unsaturated LCFA degrader) are being sequenced (DOE-Joint Genome Institute, http://www.jgi.doe.gov), and comparative genomics of these related species may shed more light on fatty-acid degrading pathways and the regulatory mechanisms that govern degradation of fatty acids. Moreover, it can give further insights in the mechanisms involved in the degradation of unsaturated LCFA.

Special reactor types and reactor operation conditions are required to optimize methane formation from LCFA by mixed methanogenic communities. The mechanism of interspecies hydrogen transfer in methanogenic communities is rather well understood, though the role of formate as a possible alternative electron carrier was never shown for LCFA degradation. LCFA-degrading communities are constrained by thermodynamic possibilities and physical aggregation of the active biomass into mixed microcolonies. The physical properties of the fatty-acid substrate might interfere with the active biomass but how that limits the kinetics of diffusion and conversion is presently unclear. Also microbial toxicity and inhibition factors associated with LCFA need to be further assessed.

Research in which the effect of sulphate on methanogenesis from LCFA was studied showed that the syntrophic LCFA-degrading bacteria are not easily out competed by sulphate-reducing bacteria with the ability to degrade LCFA. Instead, upon exposure to sulphate, the hydrogen-utilizing methanogens are replaced by hydrogen-utilizing sulphate-reducing bacteria, which can be explained by the higher affinity for hydrogen of sulphate-reducing bacteria. Additional research is needed to explore the exact niches of LCFA-degrading acetogenic and LCFA-degrading sulphate-reducing bacteria.

Overall, knowledge on microbial anaerobic LCFA degradation in anaerobic bioreactors has increased in the past years, with the clear aim of enhancing their conversion to biogas. However, insights of LCFA degradation mechanisms

might be extended to other environments, such as oil fields. Rozanova *et al.* (1997) suggested that fatty acids could be excreted by aerobic hydrocarbon-degrading bacteria present in oil wells. These fatty acids would be subsequently consumed by fatty-acid degrading methanogenic syntrophic communities. Later on, strictly anaerobic degradation of alkanes by proton-reducing bacteria in association with methanogens was found (Zengler *et al.*, 1999; Anderson & Lovley, 2000). Syntrophic bacteria from the genus *Syntrophus* and methanogenic archaea from *Methanosaeta*, *Methanospirillum* and *Methanoculleus* genera were detected in a methanogenic enrichment culture degrading hexadecane (Zengler *et al.*, 1999). The pathway of syntrophic alkane degradation is not yet clarified and the intermediaries involved in this degradation are not known. Future research is necessary to understand the physiology and ecological significance of microorganisms present in hydrocarbon-rich environments.

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