Non-syntrophic reactions in anaerobic unsaturated LCFA conversion by methanogenic sludges

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Abstract
Lipids are energy-rich compounds. This energy can be conserved as biogas in anaerobic bioreactors but the process is frequently hindered by long-chain fatty acids (LCFA) accumulation. LCFA catabolism is thought to occur via beta-oxidation, performed by anaerobic bacteria that live in obligatory syntrophy with H_2-consuming methanogens, but the initial steps of unsaturated LCFA biodegradation are still unclear. In this work we hypothesize that these initial steps do not depend on interspecies H_2 transfer. To test this, six anaerobic bioreactors were continuously fed with saturated or unsaturated C16- and C18-LCFA, and operated in the presence or absence of bromoethanesulfonate, a selective inhibitor of methanogens. Intermediates of LCFA degradation including long- and medium-chain fatty acids, volatile fatty acids and methane were monitored. Bacterial community composition was analysed in the different bioreactors by denaturing gradient gel electrophoresis of 16S rRNA reverse transcriptase-PCR products. In the presence or absence of the inhibitor of methanogenesis, palmitate (C16:0) accumulated during the degradation of oleate (C18:1), accounting for more than 50% of total accumulated LCFA. Palmitoleate (C16:1) feeding resulted in the build-up of myristate (C14:0) and palmitate (C16:0). Accumulation of saturated intermediary-LCFA was two to four times higher in bioreactors in which methanogenesis was inhibited compared to methanogenic bioreactors. Beta-oxidation of saturated intermediates only occurred in methanogenic bioreactors. No catabolic activity was observed in the bioreactor fed with saturated LCFA when methanogenesis was inhibited. These results show that the first steps of unsaturated LCFA degradation are not obligatorily syntrophic, and suggest that beta-oxidation is the limiting step in the overall conversion of LCFA to methane.

Keywords
Anaerobic bioreactors; beta-oxidation; BrES; 16S rRNA gene; syntrophy; unsaturated LCFA

INTRODUCTION

In methanogenic environments, saturated and unsaturated long-chain fatty acids (LCFA) are thought to be degraded via β-oxidation, a cyclic process in which two carbon atoms are sequentially removed per cycle, with the production of a C_{n-2} fatty acid, acetate and two moles of molecular hydrogen (Sousa et al., 2009). These reactions are only thermodynamically feasible when the hydrogen partial pressure is kept low, which is generally accomplished through syntrophic cooperation of anaerobic bacteria with hydrogenotrophic methanogenic archaea. Syntrophic partners share the energy from the partial reactions in conditions close to thermodynamic equilibrium (McInerney et al., 2008; Sousa et al., 2009; Stams et al., 2012).

The initial steps involved in the conversion of unsaturated fatty acids, however, have not been extensively studied. Weng and Jeris (1976) suggested that chain saturation is required before β-oxidation can take place, whereas other authors suggest that direct β-oxidation is also feasible (Lalman and Bagley, 2000). Unsaturated LCFA have been reported as the most toxic to the anaerobic microbial communities (Lalman and Bagley, 2002), and palmitate (a saturated C16-LCFA) was found to accumulate in bioreactors during mono-unsaturated C18-LCFA degradation (Pereira et al., 2002). This is an intriguing observation that has never been fully explained. In this

work, we studied the first steps of unsaturated LCFA degradation, hypothesizing that unsaturated LCFA conversion can occur without the need of syntrophic interactions. This hypothesis was tested in anaerobic bioreactors fed with saturated or unsaturated C16 and C18 LCFA, and operated in the presence or absence of a methanogenic activity inhibitor. Intermediary LCFA, medium-chain fatty acids (MCFA), volatile fatty acids (VFA) and methane were monitored throughout the experiment. Microbial community changes were analysed at the end of the reactors operation using a 16S rRNA gene approach.

MATERIALS AND METHODS

Bioreactors operation

Six up-flow anaerobic bioreactors were operated in continuous, at 37 °C, with different LCFA as energy and carbon source. Granular sludge, collected from a pilot-scale up-flow anaerobic sludge blanket reactor treating winery effluent, was used as inoculum at a final concentration of 10 gVS L^{-1}. Three bioreactors were inoculated with methanogenic granules (MR – methanogenic reactors) and operated in parallel with three bioreactors inoculated with granules submitted to previous treatment for methanogenesis inhibition (IR – inhibited reactors). This treatment consisted of a 48 hours batch contact with 30 mM 2-bromoethanesulfonate (BrES) solution. Sodium salts of oleate (C18:1, purum, min. 82% assay of fatty acids, Riedel-de Häen), palmitoleate (C16:1, 99%, Acros) and a mixture of stearate (C18:0) and palmitate (C16:0) (71:24% total LCFA w/w, respectively; min. 88% assay of fatty acids, Sigma-Aldrich) were used as substrates in reactors MR and IR (Table 1). The reactors were operated at organic loading rates (OLR) between 0.8 and 2.8 mol LCFA m^{-3} day^{-1} and hydraulic retention time of 2 days. IR reactors were also continuously supplied with 20 mM BrES (Table 1). Biogas production was measured with a Ritter MilliGascounter® and methane content was analysed in a Micro-GC CP-4900 (Varian Inc.) equipped with a thermal conductivity detector. Mixed liquor samples were periodically withdrawn from the reactors for VFA analysis (HPLC, Jasco, Japan) and LCFA quantification (as previously described by Neves et al., 2009).

Microbial communities profiling

At the end of the continuous operation, sludge samples were collected from MR and IR reactors fed with unsaturated LCFA. Samples were immediately suspended in RNAlater® (Sigma) and stored at -20 °C. Total RNA was extracted using the FastRNA®Pro Soil-Direct Kit (MP Biomedical), cDNA copies were synthesised using SuperScript™ reverse transcriptase III (Invitrogen) and bacterial 16S rRNA gene fragments were further amplified by PCR using the primers set U968GC-f/ L1401-r (Nübel et al., 1996). Microbial communities' profiles were studied by DGGE analysis of the PCR amplification products. DGGE profiles were compared using the Bionumerics™ software.

RESULTS AND DISCUSSION

Saturated and unsaturated LCFA were converted to methane, acetate and intermediary-LCFA in all the methanogenic reactors (MRol, MRpalmitol, MRste+pal), as shown in Table 1. Sludges submitted to selective inhibition of methanogenic activity converted unsaturated LCFA to saturated LCFA-intermediates, but these intermediates accumulated consistently achieving bulk concentrations two to four times higher than in MR reactors (Table 1). Degradation of the LCFA intermediates did not take place in the reactors in which methanogenesis was inhibited. IRste+pal reactor did not show any catalytic activity towards the saturated LCFA, and stearate and palmitate accumulated in the bulk in relative amounts similar to the feeding mixture supplied, i.e. 76±3% and 23±2% of total LCFA, respectively.

Table 1. Operational parameters, cumulative methane production and bulk fatty acids concentrations (mM) at the end of the operation time. Maximum LCFA concentrations (mM) during the whole operation time are shown in parenthesis.

	MRol	**IRol**	**MRpalmitol**	**IRpalmitol**	**MRste+pal**	**IRste+pal**
Substrate Cn:d [a]	Oleate C18:1	Oleate C18:1	Palmitoleate C16:1	Palmitoleate C16:1	Stearate+Palmitate C18:0+C16:0	Stearate+Palmitate C18:0+C16:0
BrES concentration (mM)	0	20	0	20	0	20
OLR (mol LCFA m^{-3} day^{-1})	1.62	1.62	2.75	2.75	0.81	0.81
Time of continuous operation (days)	24	24	26	26	28	28
Cumulative CH_4 production (mmol)	242	0	15	0	93	0
Theoretical CH_4 production (mmol) [b]	482	n.a.	130	n.a.	281	n.a.
Methane yield (%)	50	n.a.	11	n.a.	33	n.a.
Acetate	1.9	1.7	6.6	1.2	0.9	0.8
Myristate (C14:0)	0.1±0.0 (0.2±0.0)	0.6±0.1 (0.6±0.1)	2.5±0.8 (2.5±0.8)	3.9±0.5 (4.0±0.4)	0.0±0.0 (0.0±0.0)	0.3±0.0 (0.3±0.0)
Palmitate (C16:0)	0.8±0.0 (2.0±0.3)	7.7±0.0 (7.7±0.0)	3.6±1.3 (3.6±1.3)	2.5±0.3 (2.5±0.3)	0.2±0.1 (1.0±0.0)	4.4±0.2 (4.4±0.2)
Palmitoleate (C16:1)	0.0±0.0 (0.0±0.0)	0.0±0.0 (0.0±0.0)	2.2±0.5 (2.2±0.5)	0.5±0.1 (0.7±0.0)	0.0±0.0 (0.0±0.0)	0.0±0.0 (0.0±0.0)
Stearate (C18:0)	0.1±0.0 (0.1±0.0)	1.3±0.1 (1.3±0.1)	0.0±0.0 (0.0±0.0)	0.0±0.0 (0.0±0.0)	0.7±0.3 (3.8±0.0)	11.4±1.7 (11.4±1.7)
Oleate (C18:1)	0.1±0.0 (0.5±0.1)	1.4±0.1 (1.4±0.1)	0.0±0.0 (0.0±0.0)	0.0±0.0 (0.0±0.0)	0.0±0.0 (0.0±0.0)	0.0±0.0 (0.0±0.0)

[a] Cn:d - n is the number of carbon atoms and d the number of double bonds in the LCFA.

[b] Calculated considering the stoichiometric values of methane production from the complete conversion of each LCFA to methane.

Palmitate (C16:0) was the main intermediate of oleate (C18:1) biodegradation, accounting for more than 50% and up to 83% of total LCFA accumulated in reactors MRol and IRol. Palmitoleate (C16:1) was not detected in these reactors and stearate (C18:0) represented less that 13% of total accumulated LCFA. Degradation of mono-unsaturated C16 followed a similar pathway, causing myristate (C14:0) and palmitate to accumulate in MRpalmol and IRpalmol (Table 1).

These results show that saturated C_{n-2}-LCFA accumulate in continuous bioreactors fed with unsaturated C_n-LCFA, irrespectively whether methanogenesis takes place. Beta-oxidation of the saturated LCFA to acetate is only possible associated with methanogenic activity, indicating that the first steps of unsaturated LCFA degradation are not obligatorily syntrophic. Moreover, β-oxidation appears to be the limiting step in the overall unsaturated LCFA biodegradation. Thus, enhancement of the syntrophic interactions is probably the key for a faster and efficient methane production from these substrates.

The first steps of unsaturated LCFA biodegradation entail conjugated hydrogenation and beta-oxidation, which may be performed by one or more microorganisms. Bacterial DGGE profiles of the community developed in IR bioreactors fed with unsaturated LCFA clustered together with 72% similarity. In addition, different DGGE profiles were obtained for sludges with and without methanogenic activity (data not shown), suggesting that different microorganisms may be performing the first steps of unsaturated LCFA biodegradation and β-oxidation of saturated LCFA, respectively. Pyrosequencing-based analysis of 16S rRNA amplicons generated from cDNA of each sample is currently on going and will possibly bring new insights on the microorganisms involved in non-syntrophic LCFA degradation in anaerobic bioreactors.

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