Effect of long-chain fatty acids (LCFA) on the prevalence and viability of hydrogenotrophic methanogens

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Abstract

Anaerobic degradation of long-chain fatty acids (LCFA) is essential for efficient biogas production from complex lipid-containing wastewaters. Methanogens play a key role in this process, but the general idea is that LCFA exert a toxic effect towards these microorganisms that impairs good methane recovery. In this work, the effect of saturated (palmitate, C16:0) and unsaturated (oleate, C18:1) LCFA towards hydrogenotrophic methanogens was studied in batch enrichments and in pure cultures. *Methanospirillum hungatei* and *Methanobacterium formicicum* were added to oleate- and palmitate-degrading enrichments, cultures OM and PM, and their prevalence was subsequently monitored by PCR-DGGE. *M. formicicum* grew in both OM and PM cultures, while *M. hungatei* only prevailed in PM culture. Viability tests using live/dead staining further confirmed that *M. hungatei* is more sensitive to oleate than *M. formicicum*. The percentage of damaged cells, caused by the exposure to 0.5 mM of oleate, was higher in *M. hungatei* cultures (99 ± 1 %) than in *M. formicicum* cultures (53 ± 10 %). These results suggest that oleate is more inhibitory to methanogens than palmitate, although methane production was not completely inhibited with either LCFA.

Keywords
LCFA; Methanogens prevalence; Methanogens inhibition; Cell viability.

INTRODUCTION

Toxicity of LCFA is reported as the main reason for the insufficient treatment of LCFA-containing wastewaters (Angelidaki & Ahring 1992; Hanaki *et al.* 1981; Hwu & Lettinga 1997; Rinzema *et al.* 1994). The adverse effect of LCFA appears to be more pronounced at longer chain length and more unsaturated bonds (Demeyer & Henderic 1967; Prins *et al.* 1972). Nevertheless, methanogens are abundant in anaerobic reactors treating LCFA-containing effluents (Salvador *et al.* 2012; Sousa *et al.* 2007a). Most commonly detected genera in reactors fed with LCFA are *Methanobacterium*, *Methanosaeta*, and *Methanosarcina*; *Methanospirillum* species are occasionally detected (Sousa *et al.* 2009). The aim of this work was to clarify the effect of LCFA on hydrogenotrophic methanogens, by evaluating (i) their prevalence in LCFA-degrading enrichment cultures, and (ii) the effect of LCFA on membrane integrity of pure cultures of methanogens.

MATERIAL & METHODS

Prevalence of hydrogenotrophic methanogens in LCFA-degrading enrichments

Oleate and palmitate enrichments, cultures OM and PM, were obtained as previously described by Sousa *et al.* (2007b). *M. hungatei* (Mh) and *M. formicicum* (Mf) (5%, v/v) were separately added to OM and PM enrichments. Cultures OM-Mh/PM-Mh and OM-Mf/PM-Mf were sub-cultured with 1 mM palmitate/oleate. Samples (10 mL) were withdrawn from all the cultures before each transfer to evaluate the prevalence of hydrogenotrophic methanogens by PCR-DGGE. Total genomic DNA was extracted using a FastDNA SPIN kit for soil (MP Biomedicals, USA) and archaeal 16S rRNA

genes were amplified by PCR using the primer set A109(T)-f/515GC-r, as detailed in Salvador *et al.* (2012). DGGE analysis of the PCR products was performed by using the DCode system (Bio-Rad, Hercules, CA, USA) using a linear denaturing gradient of 30–50%, with 100% of denaturant corresponding to 7 M urea and 40% (vol/vol) formamide. Electrophoresis was performed for 16 h at 85 V and 60 ºC and DGGE gels stained with silver nitrate.

Effect of oleate on membrane integrity of hydrogentrophic methanogens

Membrane integrity of *M. hungatei* and *M. formicicum* growing on H_2/CO_2 (80:20; 1.25x105 Pa) in the presence of 0.5 and 1 mM oleate was analyzed using the LIVE/DEAD® BacLightTM bacterial viability kit according to the manufacturer's instructions (Invitrogen Molecular Probes). One mL of culture sample was stained and then filtered through 0.2 μm polycarbonate black filters (Whatman, Kent, UK). Filters were mounted with low-fluorescence immersion oil on glass microscope slides and observed on an epifluorescence microscope (Olympus BX51, x 60 magnification oil immersion objective) using FITC and Cy3 long pass filters. FITC filter reveals SYTO9 stained cells, corresponding to all cells, and Cy3 filter reveals those which are stained by both dyes and are thus damaged. From each membrane 10 to 20 randomly selected microscopic fields, each of 0.0158 mm^2, were photographed with a CCD camera (Olympus DP71) using an image acquisition software (Olympus Cell B). Images obtained with both filters, and corresponding to the same microscopic fields, were superposed and cells with damage and intact membranes were counted. All experiments were done in duplicate. Percentage of membrane-damaged cells was calculated in relation to the total number of cells.

RESULTS AND DISCUSSION

Prevalence of hydrogenotrophic methanogens in LCFA-degrading enrichments

M. formicicum prevailed in both OM and PM cultures (Fig. 1, lanes OM-Mf and PM-Mf). Conversely, *M. hungatei* could not thrive in OM enrichments, following its repeated addition to this enrichment culture (Fig. 1A, lanes OM-Mh(1) to OM-Mh(5)). In PM enrichments *M. hungatei* succeeded to co-exist with *M. formicicum*, even when *M. formicicum* was the amended methanogenic partner (Fig. 1B, lanes PM-Mf(1) to PM-Mf(4)).

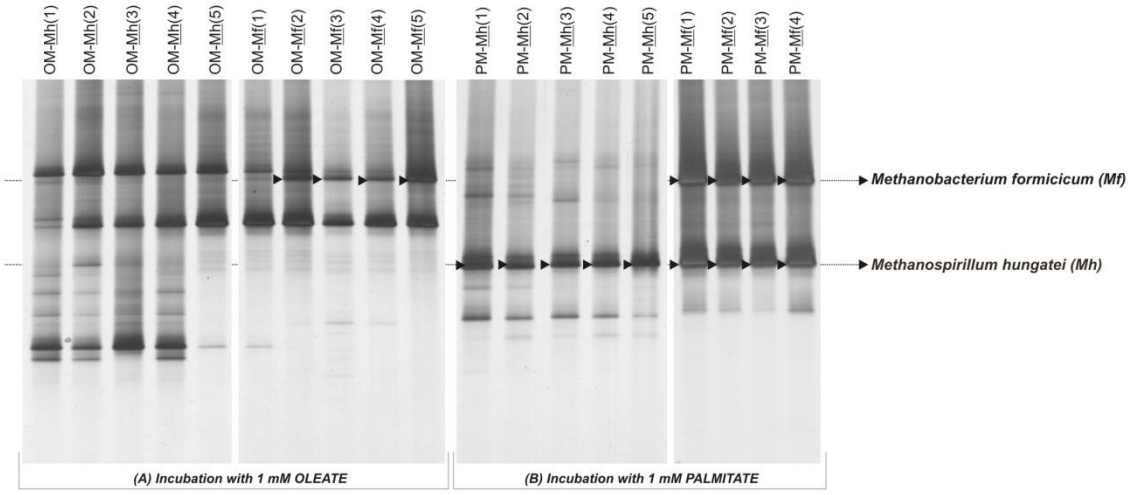


Figure 1.DGGE pattern of archaeal 16S rRNA gene fragments present in OM and PM enrichment cultures during successive transfers with addition of *Methanospirillum hungatei* (Mh) or *Methanobacterium formicicum* (Mf) as hydrogen consumers. Arrows evidence the presence of added methanogens in the enrichment cultures.

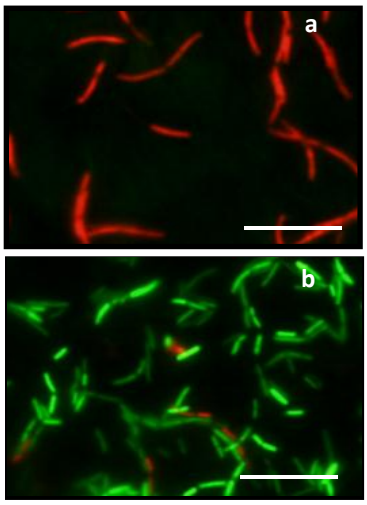
M. hungatei has been isolated from sewage sludge (Ferry *et al.* 1974) and, although frequently detected in anaerobic reactors, *Methanobacterium* species seem to have highest predominance in digesters sludge (Leclerc *et al.* 2004). Moreover, *Methanobacterium*-related microorganisms have been detected as the predominant hydrogen scavengers in oleate-contacting sludges (Pereira *et al.* 2002; Salvador *et al.* 2012; Sousa *et al.* 2007a). This fact might be related to their apparent higher resistance to withstand oleate and eventually other unsaturated LCFA.

Effect of oleate on membrane integrity of hydrogentrophic methanogens

Table 1 shows the results of membrane damaged cells of *M. hungatei* and *M. formicicum* during exposure to 0.5 and 1 mM of oleate (controls, without oleate, are also included). Membrane integrity of both *M. hungatei* and *M. formicicum* cells was affected by the presence of oleate. Incubation of *M. hungatei* cultures with 1 mM oleate resulted in a high percentage of damaged cells (99%), just after 2 h of incubation. The same percentage of *M. hungatei* membrane-damaged cells was observed after 48 h of incubation for cells exposed to 0.5 mM oleate. *M. formicicum* cells were less affected by the presence of oleate, with 37% of membrane-damaged cells after 24 h and for 0.5 mM oleate. However, 1 mM oleate and exposure time of 76 h caused 97% of cell viability loss.

Table 1. Percentage of membrane-damage cells of *M. hungatei* and *M. formicicum* growing on H_2/CO_2, with continuous exposure to 0.5 and 1 mM of oleate, after different incubation times. Control assays without the addition of oleate were also performed for *M. hungatei* and *M. formicicum.*

Oleate concentration (mM)	Percentage of membrane-damaged cells					
	M. hungatei			*M. formicicum*		
	0 h	2 h	48 h	0 h	24 h	76 h
control	5 ± 2	5 ± 4	5 ± 2	3 ± 2	2 ± 2	3 ± 2
0.5	81 ± 13	70 ± 12	99 ± 1	20 ± 11	37 ± 12	56 ± 19
1.0	89 ± 11	99 ± 1	99 ± 2	95 ± 13	82 ± 14	97 ± 4



These results show that oleate has a negative effect on membrane integrity of *M. hungatei* and *M. formicicum,* although this effect is more apparent for *M. hungatei* (Fig. 2). The susceptibility of *M. hungatei* to small concentrations of oleate is consistent with the fact that this microorganism could not endure in OM cultures. *M. hungatei* and *M. formicicum* are very similar microorganisms regarding their metabolic functions, but the properties of their membranes are distinct (Kandlera & König 1998) and this might influence the different resistance to LCFA.

Figure 2. Live/dead staining of *M. hungatei* and *M. formicicum* cells after incubation with 0.5 mM oleate for 48 hours (a) and 76 hours (b), respectively. Scale bar 10 µm.

ACKNOWLEDGEMENTS

This work was funded by FEDER funds through COMPETE program and by national funds through the Portuguese Science Foundation (FCT) in the frame of the projects FCOMP-01-0124-FEDER-007087 and FCOMP-01-0124-FEDER-014784. Financial support from FCT and the European Social Fund (ESF) through the PhD grant SFRH/BD/48960/2008 attributed to A.F. Salvador is also acknowledged.

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