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Influence of Calcium Addition on Growth of Highly Purified Syntrophic Cultures Degrading Long-Chain Fatty Acids

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Two highly purified syntrophic associations resulting in acetogenesis from stearate (SM) and oleate (OM) were obtained from the sludges of a sewage digestor. In both cases, *Methanospirillum hungatei* together with short, motile, gram-negative, nonfluorescent rods morphologically similar to *Syntrophomonas wolfei* were identified by microscopic examination. Besides growing on volatile fatty acids (butyrate through caproate), both cultures grew on oleate ($C_{18:1}$) and numerous even-numbered, saturated long-chain fatty acids (LCFA [decanoate through stearate]). In addition, during growth on LCFA, supplementation of the culture media with calcium chloride was an absolute requirement. The sole difference between the associations was observed when SM and OM cultures were transferred from a stearate to an oleate medium. The SM culture needed 10 days before starting to degrade oleate, whereas the OM culture grew immediately, but the OM culture also grew immediately when transferred to stearate medium. Saturated LCFA degradation occurred in the presence of equinormal amounts of calcium (fatty acid/Ca ratio, 2). On the other hand, OM degradation only took place in the presence of an equimolar amount of calcium (fatty acid/Ca ratio, 1). These observations are discussed by considering the solubility constants of LCFA as calcium salts and the toxicity of the free acids against microorganisms.

In methanogenic environments, long-chain fatty acids (LCFA) can be completely degraded to methane and carbon dioxide (6, 9, 12, 25). However, these compounds exhibit acute toxicity against anaerobes when their concentrations exceed a few millimoles per liter, so on a technological level, methanogenesis from LCFA is only possible in continuously fed digestors (17) in which the dilution rate is kept sufficiently low to allow the complete degradation of these compounds. The toxicity of LCFA against pure or mixed cultures appears more pronounced as the compounds become longer and more unsaturated (2, 3, 16). In addition, the sensitivity of bacteria is related to their cell wall structure, with gram-positive species and methanogens being more easily inhibited than gram-negative organisms (8, 20, 23). Fortunately, the addition of calcium reverses the inhibition of bacterial growth in batch cultures, mainly by forming insoluble salts and increasing interfacial tension (4–6).

In sewage digestors, LCFA degradation first proceeds via a β-oxidation mechanism (12, 25) leading to acetate with reductive methane. Modern concepts of anaerobic microbiology indicate that this acetogenesis is carried out by syntrophic associations of obligate hydrogen-producing bacteria and hydrogenophilic methanogens (14). In such low-sulfate media, several syntrophic associations between *Desulfovibrio* sp. and methanogens can result in an acetogenic fermentation of lactate and ethanol. However, there is no report of a syntrophic association involving a hydrogen scavenger and one of the LCFA-degrading sulfate reducers isolated so far (18), i.e., *Desulfonema magnum*, *Desulfonema limicola*, and *Desulfosarcina variabilis*. Until recently, *Syntrophomonas wolfei* (13) was the sole pure obligate hydrogen-producing bacteria that was known to oxidize fatty acids, but two other bacteria have been isolated (22), one of them morphologically similar to *S. wolfei* and the other spore forming. However, their activities are restricted to short, saturated substrates (butyrate through caproate or octanoate).

We report in this paper the establishment of highly purified syntrophic associations degrading LCFA to acetate and reductive methane. The positive effect of calcium salt additions upon LCFA oxidation in batch cultures was demonstrated.

MATERIALS AND METHODS

Sources of microorganisms. *Methanospirillum hungatei* (DSM 864) and *S. wolfei* Göttingen strain (DSM 2245B) were obtained from the German Culture Collection.

Media and conditions for growth. The anaerobic technique of Hungate as modified by Miller and Wolin (15) was used for enrichments and routine subcultures. The basal medium containing minerals and vitamins (21, 24) was buffered with PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (50 mM) and was further supplemented with yeast extract (0.3 g/liter) and rumen fluid (5%, vol/vol). Sterile stock solutions (10%, vol/vol) of fatty acid sodium salts were added aseptically to the autoclaved medium, and the subsequent addition of calcium chloride resulted in the formation of a heavy and fluffy precipitate, unless precise equimolar amounts (15 mM) of calcium and LCFA were used. Incubations were run at 35°C and pH 7.1 under an N_2/CO_2 (85:15) gas phase.

Enrichments were established by 10 successive transfers (5%, vol/vol) at 2-week intervals.

Dilution shake cultures were incubated in glass tubes (9 by 160 mm), in the presence of 0.6% agar. The media were inoculated inside an anaerobic glove box (FORMA 1020) with the simultaneous addition of an active, growing culture of *M. hungatei* (10%, vol/vol).

Analytical methods. Growth was estimated by monitoring acetate and methane production. Gas and volatile fatty acids

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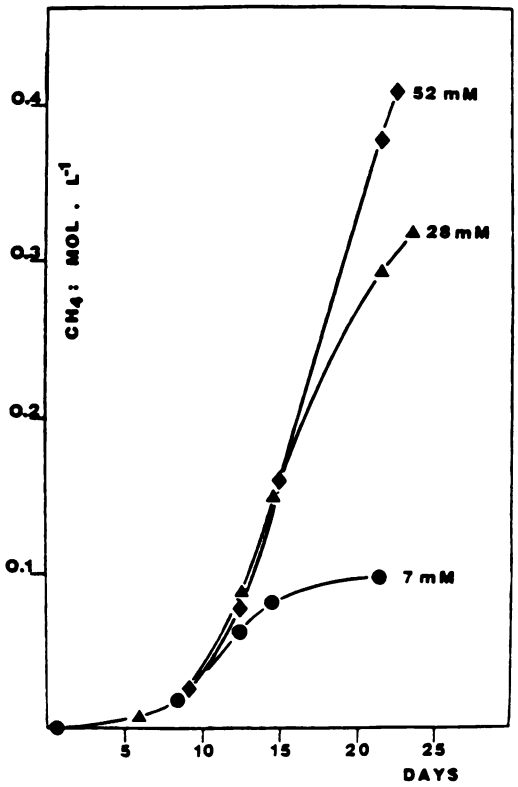

FIG. 1. Methane production from stearate by an enrichment culture in the presence of equimolar amounts of calcium. Control values were subtracted.

were determined by gas chromatography as previously described (24).

LCFA butyl esters were separated and quantified by using a stainless-steel column (1.8 m by 2 mm) packed with 28% diethylene glycol succinate on silanized Chromosorb W-AW (80-100 mesh).

Calcium was determined by atomic absorption on a PYE-UNICAM SP9 spectrophotometer, and *O*-phosphate was determined by the ascorbic acid method. Carbonates were assayed on an Oceanography International infrared carbon analyzer.

RESULTS

Establishment of highly purified acetogenic cultures degrading stearate and oleate. Two anaerobic media, one with stearate (10 mM) and one with oleate (10 mM) as main carbon and energy sources, were inoculated with sludges from a sewage digestor in Marquette-Lez-Lille, France. In batch cultures, fatty acid degradation never occurred in the absence of calcium. Equimolar additions of calcium chloride allowed good growth even when stearate and oleate concentrations up to 50 mM were reached (Fig. 1). Methane and carbon dioxide were the only end products evidenced, but a transient accumulation of acetate was sometimes observed. Experimental values were close to those of theoretical stoichiometries:

$$1 \text{ stearic acid} + 8H_2O \rightarrow 13CH_4 + 5CO_2$$
$$1 \text{ oleic acid} + 8.5H_2O \rightarrow 12.75CH_4 + 5.25CO_2$$

Microscopic examination of wet mounts revealed the presence of acetoclastic methanogens morphologically similar to *Methanosarcina mazei* (24) in the stearate enrichment culture. On the other hand, *Methanothrix*-like filamentous organisms were observed to be the prevailing methanogens in the oleate culture. After 10 successive transfers, both enrichment cultures were diluted based on the most-probable-number technique and incubated in the presence of *M. hungatei*. After 2 months, the highest dilutions permitting significant growth were 10^9 and 10^7, from the stearate and the oleate enrichment cultures, respectively. Control cultures minus LCFA did not show growth at these dilutions.

In the last tube showing positive degradation of stearate, no acetoclastic methanogens were observed. The prevailing organisms were *M. hungatei*, identified by its morphology and its autofluorescence under epi-illumination, and short, curved, motile, gram-negative, nonfluorescent rods. This culture, named SM, degraded stearate to acetate and reductive methane and was used for further studies. Similar morphologies were also observed by Lorowitz and Bryant (personal communication) in enrichment cultures degrading stearate. On the other hand, filamentous methanogens were still present in the tube corresponding to the 10^7 dilution of the oleate enrichment culture. This culture was further purified by the dilution agar shake culture technique. Incubation was performed for 6 weeks in the presence of *M. hungatei*. Clearing zones with diffuse centers were observed up to the 10^7 dilution. Careful examination showed that this center was made of numerous tiny, round colonies. One of these colonies, which degraded oleate to acetate and methane, was transferred in liquid medium inside the anaerobic glove box (OM culture). Besides *M. hungatei*, the OM culture also contained short, curved rods very close in morphology to those observed in the SM culture.

These cultures, OM and SM, did not contain acetophilic methanogens and degraded oleate and stearate by the following stoichiometries:

$$1 \text{ stearic acid} + 4CO_2 + 8H_2O \rightarrow 9 \text{ acetic acid} + 4CH_4$$
$$1 \text{ oleic acid} + 3.75CO_2 + 8.5H_2O \rightarrow 9 \text{ acetic acid} + 3.75CH_4$$

Growth properties of SM and OM cultures. Optimal conditions for growth of these two highly purified cultures were a pH of 7.1 and a temperature of 35 to 40°C. Both cultures degraded the following compounds without any lag: butyrate, valerate, caproate, and even saturated fatty acids (decanoate through octadecanoate). No growth was observed on glucose, acetate, propionate, heptanoate, octanoate, and triglycerides. Cultivation on valerate resulted in the accumulation of acetate and propionate in the medium. When transferred from saturated fatty acids to oleate medium, SM and OM cultures exhibited quite different behaviors. The latter grew immediately on this unsaturated fatty acid; however, the SM culture degraded oleate after a 10-day lag. No hydrogen sulfide was observed when the medium was supplemented with sulfate (20 mM); furthermore, bromoethanesulfonic acid (1 mM) inhibited both methanogenesis and fatty acid degradation.

Effect of calcium on the growth of SM and OM cultures. As already observed for the first enrichment and purification steps, no growth of SM and OM cultures on 15 mM LCFA concentrations occurred unless the medium was supplemented with calcium chloride. Saturated fatty acids with even numbers of carbons from decanoate to stearate were degraded when the ratio of fatty acid (millimoles added) to calcium (millimoles added) (FA/Ca) was maintained at less than 2. With these substrates, LCFA toxicity could be reversed by equinormal addition of calcium, and a shorter doubling time (Table 1) was observed on dodecanoate (lauric acid). No calcium requirement was evidenced during growth

TABLE 1. Doubling time of the OM culture growing on various fatty acids[a]

Substrate[b]	Calcium concn (mM) added	Doubling time (h)
Butyric acid (C_4)	0	27
Lauric acid (C_{12})	15	30
Myristic acid (C_{14})	15	30
Oleic acid ($C_{18:1}$)	15	40
Stearic acid (C_{18})	15	45

[a] Growth was estimated on the logarithmic sequence of CH_4 production.
[b] Concentration, 4 g of substrate per liter.

on short-chain fatty acids ranging from butyrate to caproate. This was checked by two successive transfers to avoid a possible contamination by residual LCFA or calcium present in the initial culture. Surprisingly, acetogenesis from oleate (Fig. 2) required at least an equimolar addition of calcium (FA/Ca, 1). Chemical analyses of stearate and oleate precipitates were undertaken to check a possible concurrent precipitation of calcium inorganic salts. Both precipitates were composed mainly of the fatty acids associated with calcium and phosphate; only trace amounts of carbonate were evidenced. Typical contents per 100 mg (dry weight) were as follows: 93 mg of stearate, 6 mg of Ca, and 1 mg of PO_4 (FA/Ca, 2); and 88 mg of oleate, 10 mg of Ca, and 2 mg of PO_4 (FA/Ca, 1). Despite quite different FA/Ca ratios, chemical analyses indicated only minor differences between the precipitates. Consequently, the concentrations of soluble calcium in the stearate and oleate media were 0.1 mM (FA/Ca, 2) and 3 mM (FA/Ca, 1), respectively, and in all cases more than 50% of the total phosphate ions remained in the culture supernatant. Concentrations of residual LCFA in the culture supernatant were smaller than the detection threshold of the gas-chromatographic technique.

DISCUSSION

Without calcium chloride addition, the high toxicity of LCFA with more than seven carbon atoms resulted in a complete inhibition of the growth of SM and OM cultures. After calcium addition, chemical analyses indicated very low precipitation of phosphate and carbonate; on a dry-weight basis, more than 95% of the precipitates were fatty acid calcium salt. Thus, calcium effect can be discussed by considering the solubility product constant (K_{sp}) of LCFA calcium salts. The theoretical concentrations of LCFA remaining in solution as a function of the FA/Ca ratio (Fig. 3) were calculated from the K_{sp} values reported in the literature (11). Actual concentrations were calculated from the results of calcium determination in the culture supernatant (plotted on Fig. 3). Stearate in the supernatant lies between 10^{-4} and 10^{-8} mol/liter for FA/Ca ratios ranging from 2.04 to 1.92 (Fig. 3a). Since experimentally no inhibition was evidenced with FA/Ca ratios lower than 2 and total inhibition was observed with FA/Ca ratios slightly higher than 2, even a careful adjustment of this ratio does not allow an experimental determination of an inhibition threshold. In the presence of stoichiometric amounts of calcium (FA/Ca, 2), the actual oleate and tetradecanoate concentrations in the supernatant were almost identical (2.3 μM) and 130 times higher than the soluble stearate concentration (18 nM). Under such experimental conditions, inhibition of bacterial growth was observed only with oleate. This observation confirmed the results of previous works (3, 16, 17, 20) reporting a higher toxicity of unsaturated LCFA. In our experimental conditions (substrate, 15 mM), the soluble oleate concentration has to be maintained below 0.7 μM by at least an equimolar addition of calcium salt.

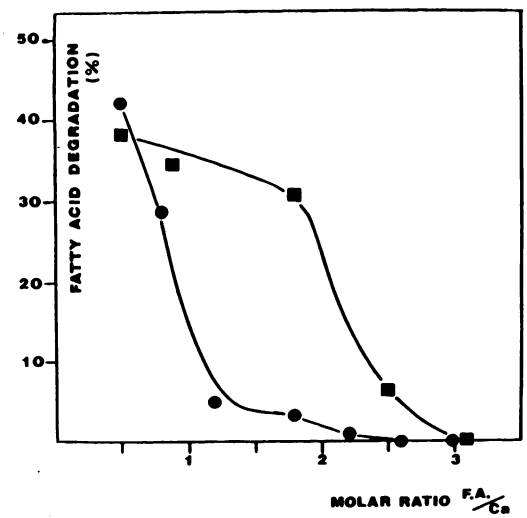

FIG. 2. Degradation of 10 mmol of oleate (●) and stearate (■) per liter by the OM consortium for various FA/Ca molar ratios. The extent of LCFA degradation was calculated from acetate and reductive methane productions after 9 days of incubation. Control values were subtracted. Percents degradation were calculated from acetate production based on the stoichiometries indicated in the text.

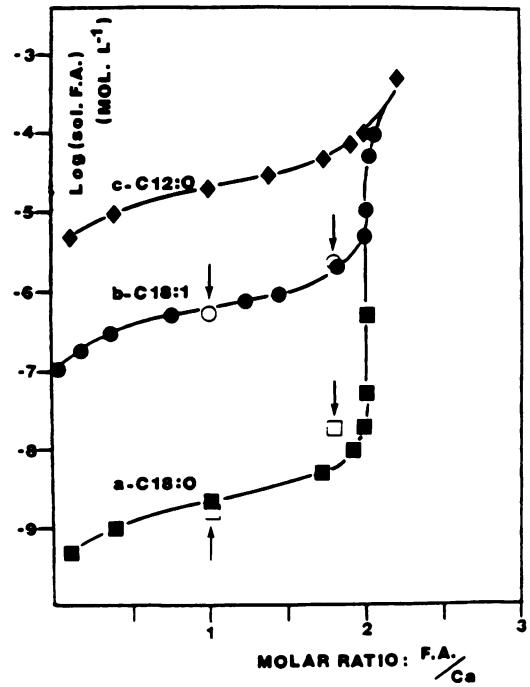

FIG. 3. Theoretical LCFA concentration in the culture supernatant (sol. F.A.) as a function of the molar ratio of total LCFA to calcium (FA/Ca), the total LCFA concentration being 15 mM. Values were calculated by the following equation:

$$FA/Ca = 2/\left(1 + \frac{2K_{sp} - SFA^3}{SFA^2 \times FA}\right)$$

where K_{sp} is the solubility product constant of the fatty acid calcium salts and SFA is the LCFA concentration in the culture supernatant. Arrows indicate actual values calculated from experimental determination of calcium in the supernatant.

Stability of morphology and immediate degradation of various saturated fatty acids indicate that SM and OM cultures have quite a wide range of substrates for growth. However, they appear dependent on lipolytic bacteria (10) such as *Anaerovibrio lipolytica* (7) for the splitting of acylglycerol. The acetate-to-methane ratios obtained after degradation of even numbered fatty acids are in good agreement with the stoichiometric balance of the β-oxidation mechanism. This is confirmed by the formation of propionate and acetate from valerate. From the present results, it is clear that the two purified cultures are syntrophic associations of LCFA-degrading acetogens together with *M. hungatei*. The stearate and the oleate organisms look very similar to *S. wolfei* (13), but they can be easily distinguished from this obligate hydrogen-producing bacteria, which is unable to perform acetogenesis from LCFA even in the presence of equimolar amounts of calcium (data not shown). Up to now only a minor difference was evidenced between SM and OM cultures when stearate-grown cells were transferred to oleate. Finally, further purification and characterization of the two organisms are being achieved to describe their taxonomic position and their physiological properties.

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