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Methane production from propionate by methanogenic mixed culture

(Anaerobic digestion; propionate; methanogenesis; mixed culture; sulfide inhibition)

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1. SUMMARY

The association of *Desulfobulbus* sp. with *Methanosarcina barkeri* 227 was able to produce CH_4 from propionate in the presence of sulfate, if a sufficient amount of ferrous iron was added to the media in order to trap the soluble sulfides produced from sulfate. In the absence of ferrous iron, soluble sulfides inhibited the acetoclastic reaction. Attempts to cultivate *Desulfobulbus* sp. with H_2-utilising methanogenic bacteria in the absence of sulfate did not succeed.

2. INTRODUCTION

Anaerobic degradation of organic matter to CH_4 proceeds through the formation of volatile fatty acids (VFA) [1,2] which are in turn transformed to the major methanogenic substrates (acetate, H_2-CO_2) mainly by obligate hydrogen-producing acetogenic bacteria (OHPAB) [2–7]. Use of propionate, one of the major VFAs formed, is of interest. Furthermore, propionate accumulates under overload conditions which often lead to digester failure.

Two types of bacteria are known to perform incomplete propionate oxidation in anaerobic environments: a sulfate-reducing bacterium *Desulfobulbus propionicus* [8] and an OHPAB *Syntrophobacter wolinii* [3]. In parallel to the microbiological characterizations of propionate degraders, some authors gave biochemical data on turn-over rates and pathways of propionate degradation [4–6]. Recently, evidence for the existence of two sub-groups of propionate users in a sulfate-limited digester was found by kinetic analysis [9]. The first sub-group, with a μ_{max} of 0.0054 h^{-1}, was reported as being similar to *S. wolinii*, the second was a faster growing sub-group with a μ_{max} of 0.05 h^{-1}, and not related to a known bacterium.

There are no reports to date of *D. propionicus* growing in syntrophic relation with hydrogenophilic bacterium in the absence of sulfate. A sulfate-reducing bacterium isolated in our laboratory, not identical to *D. propionicus*, has a μ_{max} of 0.025 h^{-1} while growing on propionate plus sulfate, and is able to use H_2 as electron donor. This paper describes attempts to create methanogenic cocultures of this propionate user with hydrogenophilic or acetoclastic methanogenic bacteria.

3. MATERIALS AND METHODS

3.1. Organisms and growth conditions

The anaerobic techniques described by Hungate

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[10] and Balch et al. [11,12] were used throughout this study. Bacteria were grown anaerobically in aluminium-seal culture tubes (Bellco Glass, Inc., Vineland, NJ). *Methanobacterium formicicum* MF was grown at 37°C in the medium of Balch et al. under 30 psi of H_2-CO_2 (4 : 1). *M. barkeri* 227 was grown at 37°C under 5 psi of N_2-CO_2 (4 : 1) in the medium described elsewhere [13] but with the TES-buffer replaced by sodium bicarbonate (5 g/l).

Desulfobulbus sp. was isolated in our laboratory from a digester and grown at 30°C under 5 psi of N_2-CO_2 (4 : 1) in a medium containing the following constituents (per l distilled water): mineral solution no. 1 [12], 50 ml; mineral solution no. 2 [12], 50 ml; trace vitamin solution [14], 10 ml; trace mineral solution [14], 10 ml; $NiCl_2 \cdot 6H_2O$, 0.5 mg; Na_2SeO_4, 3.7 mg; yeast extract, 0.1 mg; $FeSO_4 \cdot 7H_2O$, 2 mg; resazurin, 1 mg; $NaHCO_3$, 5 g; NH_4Cl, 1 g; sodium propionate, 1 g; Na_2SO_4, 3 g.

Anoxic medium was prepared by boiling the medium without added reducing agents under a stream of N_2 for 60 s. Then cysteine-HCl $\cdot$ H_2O was added at 0.5 g/l and the medium was dispensed inside an anaerobic glove box (La Calhène, Bezons, France). Media were sterilized at 120°C for 20 min. Sterile $Na_2S \cdot 9H_2O$ was added to each liquid culture before inoculation to a final concentration of 0.05% (w/v), except for the medium of *Desulfobulbus* sp. (final concentration 0.2 g/l).

3.2. Analytical procedures

Methane was analyzed in a Varian Aerograph 2700 equipped with a flame ionization detector and a 2 m stainless steel column packed with Porapack Q (80/100 mesh) and operated at 160°C. The carrier gas was N_2 at a flow rate of 20 ml/min. CH_4 was quantitated by comparing the peak height with known standards. Acetate and propionate were analysed by gas chromatography, using the same apparatus as for CH_4. A $\frac{1}{8}''$ × 2 m stainless steel column packed with 25% NPGA and 2% H_3PO_4 on Chromosorb WAW (80/100 mesh) was used. The carrier gas was N_2 saturated with formic acid at a flow rate of 30 ml/min and operated at 160°C.

Each liquid sample (1.0 ml) was removed aseptically and 20 μl of H_3PO_4 (50% v/v) were added. Then the sample was centrifuged for 5 min at 10 000 × *g* to remove cells. The peak height was compared to known standards. Soluble sulfides were estimated according to a new method (F. Widdel and R. Cordruwisch, in press).

3.3. Experimental conditions

The basal medium used for all experiments was the same as described for the culture of *Desulfobulbus* sp., except for Na_2SO_4 and sodium propionate. Sulfate and propionate were added to final concentrations of 0.9 g/l and 0.6 g/l. All experiments were run in triplicate. Controls without substrates were made each time.

4. RESULTS AND DISCUSSION

Bryant et al. [15] and McInerney and Bryant [16] described the coupling by interspecies H_2 transfer between *Desulfovibrio* sp. and an H_2-using methanogenic bacterium in a sulfate-free medium with lactate or ethanol as substrate. The propionate degrader, *Desulfobulbus* sp., is able to use H_2 as electron donor in the presence of sulfate. Therefore, the strain was tested for the ability to evolve hydrogen in a syntrophic association with the hydrogenophilic bacteria *M. formicicum* MF or *M. barkeri* 227. Attempts to couple these bacteria in a sulfate-free medium failed. Thus *Desulfobulbus* sp. cannot be a representative of the second sub-

Table 1

End products formed after different periods of incubation of two mixed cultures

mmol/l	6 days [a]	13 days [a]	13 days [b]
Propionate	0	0	6.24
Acetate	6.20	6.25	0
CH_4	0	0	0
Total sulfides	nd [c]	5	nd

[a] Association of *Desulfobulbus* sp. with *M. barkeri* 227 on propionate (6.24 mM) in presence of sulfate (6.3 mM).

[b] Association of *Desulfobulbus* sp. with either *M. barkeri* 227 or *M. formicicum* MF on propionate (6.24 mM) without sulfate or other electron acceptor (all sulfate salts in the medium were replaced by their chloride equivalents).

[c] not determined.

Table 2

CH_4 production by *M. barkeri* 227 at 30°C from sodium acetate (1 g/l)

	mmol CH_4/l/day	final CH_4 mmol/l
A	0.85	6.6
B	0.85	6.6
C	0.80	6.8

A, acetate only; B, in presence of Na_2SO_4 (6.3 mM); C, in presence of sodium propionate (6.24 mM).

group of propionate users reported by Heyes and Hall [9].

CH_4 could also be produced from propionate by the coculture *Desulfobulbus* sp. and *M. barkeri* 227. Under those conditions propionate was converted to acetate plus CO_2, and no CH_4 was evolved (Table 1). A pure culture of *M. barkeri* 227 was used to test for inhibition of the acetoclastic reaction by either propionate or sulfate. No inhibition was found (Table 2), thus the inhibition was believed to be caused by sulfides. The association was therefore grown in a medium supplemented with an excess of iron sulfate, to trap soluble sulfide as FeS.

After 10 days of incubation, all propionate was converted to acetate and CO_2, and the acetate to CH_4 and CO_2. No soluble sulfide was detected (Table 3; Fig. 1). So, in digesters or other anaerobic ecosystems, where acetate is not limiting [17], such a pathway for propionate degradation could be expected. The anaerobic environment must contain sufficient amounts of sulfate and iron, or other metallic ions able to trap soluble sulfide.

Table 3

End products formed by the association *Desulfobulbus* sp. - *M. barkeri* 227 grown on propionate (6.2 mM) plus sulfate (6.3 mM) with 6.5 mM of $FeSO_4 \cdot 7H_2O$

mmol/l	6 days	10 days
Propionate	0	0
Acetate	3.07	0
Sulfides	nd [a]	0
CH_4	3.05	6.20

[a] nd, not determined.

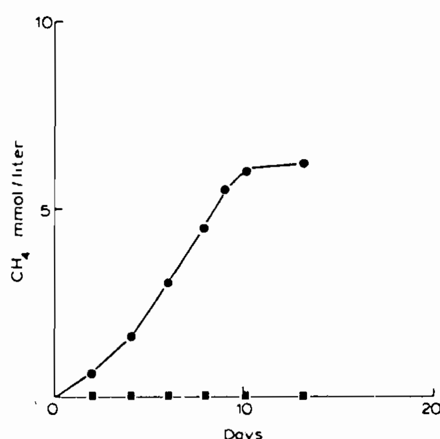

Fig. 1. Methane production by the association *Desulfobulbus* sp. and *M. barkeri* 227 grown on propionate and sulfate, in the presence of 6.5 mM $FeSO_4 \cdot 7H_2O$ ●, or in the absence of iron ■.

Furthermore, even when trapped as FeS, sulfide is probably still available to methanogens, as shown by Scherer et al. [18] with *M. barkeri* grown on methanol. The fact that iron must be added in order to avoid sulfide inhibition is supported by, and appears to explain, the results obtained by Van Den Berg et al. [19], who noted that the addition of 5 mM of ferrous chloride to a fermenter, fed with bean-blanching waste, greatly stimulated the acetate conversion.

In the literature there is a lack of data about relative cell numbers of sulfate-reducing bacteria and OHPAB using propionate in digesters working with different types of waste. Nevertheless, the activity of bacteria such as *D. propionicus* in anaerobic digesters is probably of little importance in comparison to OHPAB activity. Thus, according to the data of Heyes and Hall [9], other OHPAB with faster growth rates than *S. wolinii* must be present.

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