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Cultivation of syntrophic anaerobic bacteria in membrane-separated culture devices

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1. SUMMARY

A dialysis culture device was used for growth of syntrophic fatty acid-oxidizing and ethanol-oxidizing anaerobic bacteria. A pure culture of the fatty acid oxidizer *Clostridium bryantii* was grown inside dialysis tubing which was surrounded by a pure culture of *Desulfovibrio vulgaris*. The same apparatus was used for the syntrophic cultivation of *Pelobacter acetylenicus* and *Acetobacterium woodii* with ethanol as substrate. In both cases, substrate degradation and product formation were about half as fast as with the homogeneously mixed control cultures. In the compartment of the hydrogen producer, the concentration of free hydrogen during syntrophic ethanol degradation was about 10 times as high as in that of the hydrogen utilizer, whereas the homogeneously mixed culture exhibited an intermediate hydrogen partial pressure.

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2. INTRODUCTION

Fatty acids of 3 or more carbon atoms, as well as alcohols of 2 or more carbon atoms, are degraded in methanogenic environments by the cooperation of fermenting and methanogenic bacteria. In both cases, the substrate is oxidized by fermenting bacteria to acetate, other fatty acids, or carbon dioxide, and the reducing equivalents released are transferred to the methanogenic partner organism, most probably in the form of molecular hydrogen. Because the primary oxidation reaction is endergonic under standard conditions, the fermenting bacteria cannot be grown in pure culture but depend on the presence of a hydrogen-scavenging partner for this interspecies hydrogen transfer [1–4]. Defined cocultures of ethanol-oxidizing [5,6], propionate-oxidizing [7], and butyrate-oxidizing anaerobes [8–10] have recently been isolated. Several syntrophically ethanol-oxidizing bacteria can be grown in pure culture with acetaldehyde analogues as substrates [6,11–13]. A similar approach allowed growth of *Syntrophomonas wolinii* in pure culture with crotonate as substrate (P.S. Beaty and M.J. McInerney Abstr. Ann. Meetg. Am. Soc. Microbiol. I-132, Washington, DC, 1986), but enrichments

with crotonate or 3-hydroxybutyrate did not yield syntrophically fatty acid-oxidizing anaerobes [14].

In the present study, the dialysis culture method [15] was applied to examine whether syntrophic partner organisms can be grown in a membrane-separated culture device, and to what extent the separating membrane affects the interspecies hydrogen transfer kinetics.

3. MATERIALS AND METHODS

3.1. Bacterial strains

The following strains were used for coculture studies. *A. woodii* DSM1030 is a homoacetogenic bacterium which cannot utilize ethanol as substrate [16]. *P. acetylenicus* strain WoAcyl DSM3246 is a strict anaerobe which was isolated with acetylene as substrate and can degrade ethanol in syntrophic culture with, e.g., *A. woodii* [13]. *C. bryantii* strain CuCa1, DSM3014, is a marine obligately syntrophic fatty acid-oxidizing bacterium [9]. *Desulfovibrio* sp. strain E70 is a marine sulfate-reducing isolate which utilizes lactate, pyruvate and formate as electron donors. It was isolated and kindly provided by Dr. F. Widdel, Konstanz.

3.2. Cultivation and chemical analyses

All procedures for cultivation were as previously described [14,17,18]. The mineral medium for cultivation was carbonate-buffered and sulfide-reduced, and contained trace element solution SL 10. The pH was adjusted to 7.2–7.4. Pure and non-separated mixed cultures were grown in 50-ml screw-cap bottles.

Growth experiments in membrane-separated cultures were carried out in a special cultivation device (Fig. 1). A sterile 250-ml glass bottle carrying an open screw-cap tube with a butyl rubber septum at the bottom was used as culture bottle. This was closed, without head-space, with a pierced butyl rubber stopper holding a second screw-cap tube with butyl rubber septum. Dialysis tubing ('Servapor', Serva, Heidelberg) closed at one end with a tubing clamp, was fitted tightly around the rubber stopper.

For the fatty acid-oxidizing coculture, the dialysis tube was filled with a pasteurized (20 min at 80°C) culture of sporulated cells of *C. bryantii*, whereas the glass bottle was filled with salt-water mineral medium containing 10 mM caproate, 20 mM sulfate, and *Desulfovibrio* sp. For the ethanol-degrading coculture, the bottle was filled with freshwater mineral medium containing 10 mM ethanol and 50 ml of a *A. woodii* culture. The tubing compartment was inoculated with a sample of a *P. acetylenicus* culture. Samples for analysis of substrates and products were taken at intervals by syringes. 1 ml of fresh medium was added at one sampling port, and the same volume was taken by another syringe from the other sampling port. The culture was mixed thoroughly between both samplings in every case. Samples were extracted for dissolved hydrogen in nitrogen-flushed 12-ml tubes containing 50 μl of 10 N formic acid, and sealed with butyl rubber septa. Ethanol and acetate were assayed by standard gas chromatography procedures [18]. Hydrogen in the gas phase was quantified by a hydrogen analyzer based on the HgO-to-Hg vapor conversion technique [19] with a detection limit of 10 $nl \cdot l^{-1}$ (0.5 nM) dissolved hydrogen. All determinations were run in duplicate. All chemicals were of reagent grade and were obtained from Fluka, Neu-Ulm, F.R.G. or Serva , Heidelberg, F.R.G.

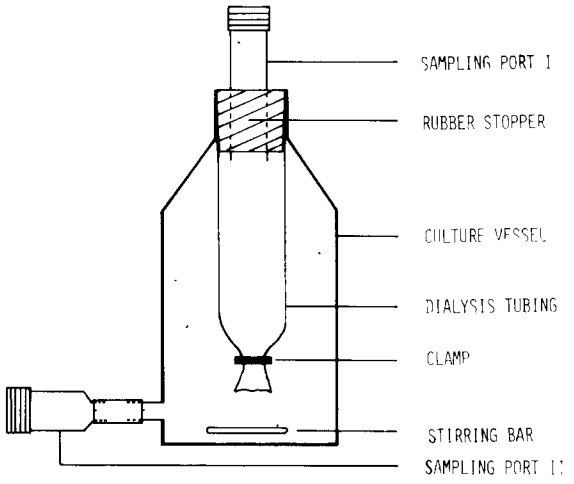

Fig. 1 Culture bottle for cocultivation of syntrophic partner bacteria separated by a dialysis membrane

4. RESULTS

C. bryantii is an obligately syntrophic fatty acid-oxidizing bacterium which forms spores in the stationary growth phase. It therefore lends itself to growth experiments in dialysis cocultures because an inoculum of viable spores devoid of living vegetative partner cells can be prepared by pasteurization. In our experiments we chose a combination with a sulfate-reducing hydrogen scavenger in the outer compartment of the dialysis culture bottle, because sulfate reducers are very efficient hydrogen scavengers and can be cultivated without a gas headspace.

A pure culture of *C. bryantii* grown in dialysis culture is depicted in Fig. 2. It appears that this strain, also in pure culture, forms cell aggregates of various sizes, a property that might be of importance in interspecies hydrogen transfer in natural communities. The doubling time of the membrane-separated coculture was about 6 days, as estimated from the kinetics of acetate and sulfide formation (data not shown). This is about twice the doubling time of the homogeneous mixed culture (3 days [9]).

Experiments on growth and hydrogen transfer kinetics were carried out with an ethanol-degrading coculture of *P. acetylenicus* and *A. woodii*. A combination with a homoacetogenic bacterium was chosen again to allow cocultivation without a gas headspace. Fig. 3 shows a time course of substrate utilization, product formation and hydrogen concentration in a homogeneously mixed coculture. Here as well as in the membrane-separated coculture, the expected final acetate concentration of 15 mM is not reached due to the dilution by fresh medium during sampling. The doubling time of acetate formation was about 7.2 hours. The concentration of dissolved hydrogen varied between 120 $nl \cdot ml^{-1}$ (5 μM) at the beginning and 0.1 $nl \cdot ml^{-1}$ (40 nM) at the end of growth. In the membrane-separated coculture, the doubling time of acetate formation was 14–18 h in both the inner and the outer compartment, and correlated well with ethanol consumption (Fig. 4). The hydrogen concentration in the compartment of the hydrogen producer (inside) increased at the beginning up to 580 $nl \cdot ml^{-1}$ (24 μM) and dropped

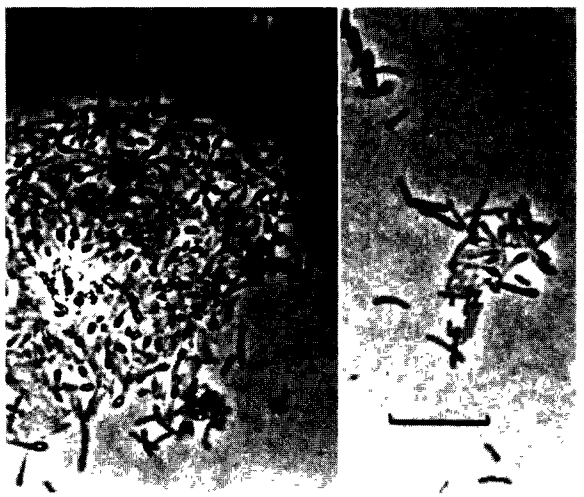

Fig. 2. Phase-contrast photomicrograph of *Clostridium bryantii* strain CuCa1 grown in pure culture in the dialysis tube Bar = 10 μm.

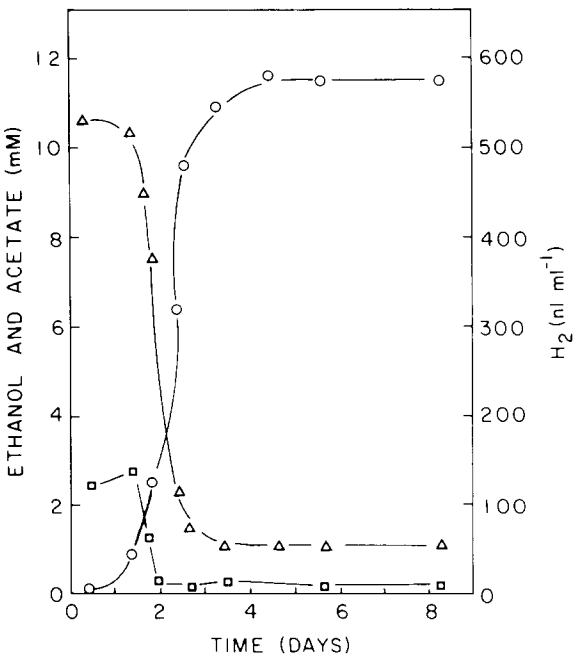

Fig. 3. Time course of ethanol fermentation to acetate by a homogeneously mixed culture of *Pelobacter acetylenicus* and *Acetobacterium woodii* △, Ethanol; ○, acetate; □, dissolved hydrogen.

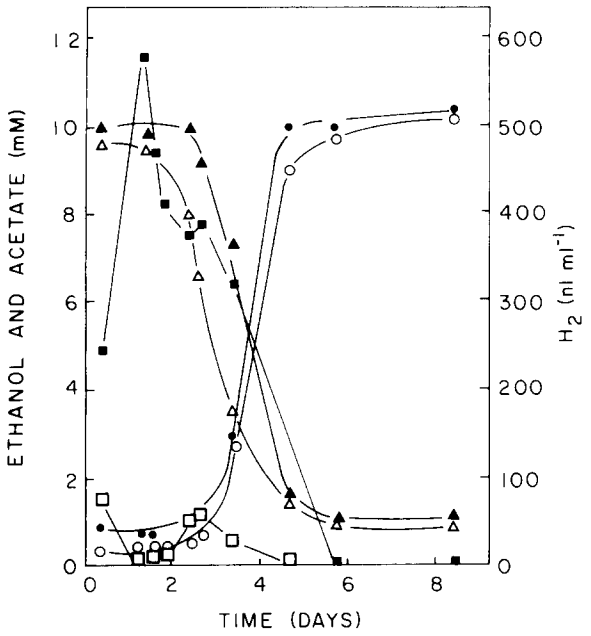

Fig. 4 Time course of ethanol fermentation to acetate in the membrane-separated dialysis culture. The inner compartment was inoculated with *Pelobacter acetylenicus* (closed symbols), the outer one with *Acetobacterium woodii* (open symbols). (△, ▲), Ethanol; (○, ●), acetate; (□, ■), dissolved hydrogen.

during growth to a final value of 0.1 nl · ml^{-1} (40 nM). In the compartment of the hydrogen consumer (outside), the hydrogen concentration never exceeded 75 nl · ml^{-1} (3 μM) and reached the same final value as in the inner compartment.

5. DISCUSSION

Both the oxidation of ethanol to acetate or of butyrate to 2 acetate with concomitant reduction of protons to hydrogen are endergonic fermentation reactions under standard conditions at pH 7.0 (calculations after [20]):

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2\ H_2$$

$$\Delta G_0' = +9.7\ \text{kJ} \cdot \text{mol}^{-1}$$

$$CH_3CH_2CH_2COO^- + 2\ H_2O \rightarrow 2\ CH_3COO^- + H^+ + 2\ H_2$$

$$\Delta G_0' = +48.3\ \text{kJ} \cdot \text{mol}^{-1}$$

Both can become exergonic only if the hydrogen partial pressure is kept low enough, e.g., by the activity of hydrogen-scavenging anaerobes. In the present study, ethanol oxidation was combined with homoacetogenic reduction of carbon dioxide, and fatty acid oxidation with sulfate reduction.

$$2\ CH_3CH_2OH + 2\ CO_2 \rightarrow 3\ CH_3COO^- + 3\ H^+$$

$$\Delta G_0' = -46.2\ \text{kJ} \cdot \text{mol ethanol}^{-1}$$

$$2\ CH_3CH_2COO^- + SO_4^{2-} \rightarrow 4\ CH_3COO^- + HS^- + H^+$$

$$\Delta G_0' = -27.8\ \text{kJ} \cdot \text{mol butyrate}^{-1}$$

Thus, the energetics of syntrophic alcohol oxidation are by far more favourable than those of fatty acid oxidation. From the biochemical point of view, this can be explained by the electron potentials of the oxidation reactions involved. Ethanol oxidation to acetate via acetaldehyde [6,13,21,22] releases electrons at the redox potential (E_0') of −197 mV and −540 mV, respectively. The former need a hydrogen partial pressure of 10^{-4} bar to be released as molecular hydrogen ($E_0' = -420$ mV). The situation is by far more complex with butyrate oxidation, because the electrons released during oxidation via crotonyl CoA and acetoacetyl CoA [23] arise at redox potentials of $E_0' = -15$ and −190 mV, respectively. The former especially would present extreme difficulties in reducing protons to molecular hydrogen, and it has been suggested that the fatty acid-oxidizing bacterium has to expend energy in a reversed electron transport chain to bring about proton reduction [24]. It should be mentioned that it has never been proven reliably that the electron carrier between the partners is hydrogen rather than formate, and most descriptions of defined cocultures have left this question open (see e.g., [5,8]). The redox potentials of both, however, are about the same under standard conditions, and molecular hydrogen is certainly a kinetically more efficient electron carrier than the charged formate anion. Isolation of obligately syntrophic fatty acid-oxidizing spore-formers [9] and cultivation of syntrophic ethanol-oxidizing anaerobes, the so-called 'S-organisms' [5], in pure culture with acetaldehyde analogues as substrates [6,11–13], provided an ap-

proach to growing these bacteria in cultures separated from their syntrophic partners by a dialysis membrane [15].

The cocultivation of ethanol- and butyrate-oxidizing mixed cultures in membrane-separated cocultures in the present study clearly illustrates that the transfer of reducing equivalents from one species to the other one does not necessarily require immediate contact of the partner organisms. Therefore, a direct electron transfer via 'electricity' from cell to cell can be definitely ruled out. Even the syntrophic caproate oxidation system operated sufficiently well in our culture device, although the distance between the two partners was at least equal to the thickness of the dialysis tubing, i.e., approx. 40 μM.

With the ethanol-degrading coculture, it could be demonstrated that the hydrogen concentration in the compartment of the ethanol oxidizer during the exponential growth phase was about 10 times as high as in the compartment of the homoacetogen. This finding corroborates the hypothesis that actually hydrogen is the electron transfer metabolite between the two partner organisms in this case.

The fact that in both the caproate- and the ethanol-oxidizing dialysis cocultures the doubling times were about twice as long as those of the homogeneously mixed control cocultures, demonstrates that hydrogen transfer between the partner organisms is really the rate-limiting step in substrate oxidation in both cases. This is undoubtedly due to the longer diffusion distances between the partner organisms. As a consequence, this would mean that the kinetics of growth and substrate conversion at both sides of the membrane would ultimately turn into a linear function of time the constant of which would only depend on the area and thickness of the dialysis tubing, according to Fick's law. Because the efficiency of interspecies metabolite transfer between partner organisms decreases with the third power of their relative distance to each other, it is conceivable that close contact between both partners is preferred in close-to-nature environments. The pure culture of *C. bryantii* obtained in this study shows a definite tendency to form clumps, a property that might be of essential importance for the establishment of mixed colonies. Evidence for juxtapositioning in sediments and sewage sludge was recently obtained from measurements of hydrogen fluxes [25] and in situ metabolite concentrations [26] in methanogenic freshwater environments. As a consequence, growth rates of homogeneously mixed syntrophic cocultures would not be constant but a function of cell density. Experiments on this problem with defined cocultures are in progress in our lab.

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