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Anaerobic Degradation of Propionate by a Mesophilic Acetogenic Bacterium in Coculture and Triculture with Different Methanogens

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A mesophilic acetogenic bacterium (MPOB) oxidized propionate to acetate and CO_2 in cocultures with the formate- and hydrogen-utilizing methanogens *Methanospirillum hungatei* and *Methanobacterium formicicum*. Propionate oxidation did not occur in cocultures with two *Methanobrevibacter* strains, which grew only with hydrogen. Tricultures consisting of MPOB, one of the *Methanobrevibacter* strains, and organisms which are able to convert formate into H_2 plus CO_2 (*Desulfovibrio* strain G11 or the homoacetogenic bacterium EE121) also degraded propionate. The MPOB, in the absence of methanogens, was able to couple propionate conversion to fumarate reduction. This propionate conversion was inhibited by hydrogen and by formate. Formate and hydrogen blocked the energetically unfavorable succinate oxidation to fumarate involved in propionate catabolism. Low formate and hydrogen concentrations are required for the syntrophic degradation of propionate by MPOB. In triculture with *Methanospirillum hungatei* and the aceticlastic *Methanothrix soehngenii*, propionate was degraded faster than in biculture with *Methanospirillum hungatei*, indicating that low acetate concentrations are favorable for propionate oxidation as well.

Propionate is an important intermediate in the degradation of organic matter under methanogenic conditions. It may account for 35% of the total methanogenesis in digestors (18). Propionate oxidation is accomplished by syntrophic consortia of acetogenic and methanogenic bacteria (3, 6, 18, 24). Because of unfavorable energetics propionate oxidation to acetate is only possible when the concentrations of products hydrogen and/or formate (Table 1, equations 1 and 2) are kept low by methanogens (Table 1, equations 3 and 4). At present, the relative importance of formate and hydrogen in syntrophic degradation is not clear. Several syntrophic cocultures in which only H_2-consuming methanogens were present have been described (3, 17, 24, 26). In these cocultures H_2 inhibited the degradation (1, 9). However, some observations made concerning mixed populations could not easily be explained by interspecies hydrogen transfer. Thiele and Zeikus (30) put forward the theory that interspecies formate transfer might be important as well. Boone et al. (4) investigated a butyrate-oxidizing coculture of *Syntrophomonas wolfei* and *Methanobacterium formicicum* and calculated by using a formate and hydrogen diffusion model that the high methane formation rate could not be explained by interspecies H_2 transfer but that it could be explained by interspecies formate transfer. As H_2 and formate concentrations in methanogenic ecosystems are extremely low, direct evidence for the quantitative importance of H_2 or formate transfer is difficult to obtain in mixed populations.

Recently, we succeeded in growing a mesophilic propionate-oxidizing bacterium (MPOB) on fumarate in the absence of methanogens (27). The bacterium is able to oxidize propionate to acetate and to couple this oxidation to the reduction of fumarate to succinate. In addition, the bacterium can ferment fumarate to succinate and CO_2 or reduce fumarate to succinate with hydrogen and formate as electron donors. The ability of the bacterium to grow in the absence of methanogens enabled us to construct different defined cocultures and tricultures. By using methanogens that can use both hydrogen and formate and methanogens that can use only hydrogen, the roles of hydrogen and formate in the syntrophic degradation of propionate could be studied directly. In this investigation we also used organisms that are able to interconvert hydrogen and formate (Table 1, equation 6). For this purpose we chose a homoacetogen and a *Desulfovibrio* sp. The effect of the presence of the aceticlastic *Methanothrix soehngenii* on syntrophic degradation of propionate was studied as well.

MATERIALS AND METHODS

Organisms. The MPOB was described before (21, 27). *Methanospirillum hungatei* DSM 864 (8), *Methanobacterium formicicum* DSM 1535 (5), and the *Methanobrevibacter arboriphilus* strains DSM 1125 (32) and DSM 744 (31) were purchased from the German Collection of Microorganisms (Braunschweig, Germany). *Methanothrix soehngenii* (*Methanosaeta concilii*) (14) was from our culture collection. *Desulfovibrio* strain G11 (3, 19) was isolated by us from a coculture with *Syntrophobacter wolinii* DSM 2805. The homoacetogenic bacterium EE121 was isolated at our laboratory (22).

Media and cultivation. The MPOB was routinely grown in a mineral medium containing sodium fumarate (6.5 g/liter) as the sole carbon and energy source under a gas phase of 162 kPa of N_2-CO_2 (80/20) (27). Except for *Methanothrix soehngenii*, all methanogens and *Desulfovibrio* strain G11 and the homoacetogen EE121 were cultivated in a basal mineral medium with a composition described previously (12). However, the trace minerals Na_2SeO_3 and Na_2WO_4 were omitted, and 0.25 g of cysteine, 0.25 g of yeast extract, 0.25 g of biotrypticase, and 0.16 g of sodium acetate were added per liter of medium. The gas phase was 162 kPa of H_2-CO_2 (80/20). For the routine growth of *Desulfovibrio* strain G11, 2.84 g of

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TABLE 1. Reactions possibly involved in the syntrophic degradation of propionate

Reaction equation	$\Delta G^{\circ\prime}$ (kJ/mol)[a]
Propionate oxidation	
1. $CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + 3H_2 + H^+$	+76.5
2. $CH_3CH_2COO^- + 2HCO_3^- \rightarrow CH_3COO^- + 3HCOO^- + H^+$	+72.4
Methanogenesis	
3. $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−135.6
4. $4HCOO^- + H^+ + H_2O \rightarrow CH_4 + 3HCO_3^-$	−130.1
5. $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	−31
Formate conversion	
6. $HCOO^- + H_2O \rightarrow H_2 + HCO_3^-$	+1.3

[a] $\Delta G^{\circ\prime}$ values were from reference 28.

sodium sulfate per liter was added to the medium. The homoacetogen EE121 was routinely grown on glucose (5 g/liter) under a gas phase of N_2-CO_2 (80/20). For the triculture experiment, *Desulfovibrio* strain G11 and the homoacetogen EE121 were grown in medium containing 1.7 g of formate per liter under N_2-CO_2 (80/20). *Methanothrix soehngenii* was grown in the same medium as the MPOB except that a 10-fold-higher concentration of vitamins and a 2-fold-higher concentration of trace elements were used; sodium acetate (5 g/liter) was the carbon source. All the bacteria were cultivated in 50 ml of medium in 120-ml serum vials (Aluglas Verenigde Bedrijven B. V., Amersfoort, The Netherlands) which were closed with butyl rubber stoppers (Rubber B. V., Hilversum, The Netherlands) and aluminum caps. The incubation temperature was 36 ± 1°C.

Propionate oxidation by bicultures and tricultures. Bacterial cultures were grown in the media described above, and when growth had ceased, cocultures were constructed by aseptically mixing the MPOB culture and the different hydrogen- and acetate-degrading cultures. The inoculation sizes are given below. Unless stated otherwise, propionate was added as the carbon and energy source, and the gas phase was N_2-CO_2 (80/20). At different time intervals gas and liquid samples were taken and analyzed. Propionate degradation rates were estimated from the linear parts of the propionate degradation curves.

Effect of H_2 and formate on the growth of MPOB in the absence of methanogens. MPOB were cultivated in six 120-ml serum vials containing 50 ml of mineral medium with 40 mM fumarate and 20 mM propionate as substrates and a gas phase of N_2-CO_2 (80/20). After about 1 month of incubation, two of the vials were flushed with 162 kPa of H_2-CO_2 (80/20), whereas to two other vials formate (final concentration, 20 mM) was added. Two vials served as controls. Incubation was continued, and after various periods of time samples were taken and analyzed.

Analytical methods. Methane and hydrogen were measured on a Packard-Becker 417 gas chromatograph as described before (11). Formate, malate, fumarate, succinate, acetate, and propionate were measured by high-performance liquid chromatography (7).

RESULTS

Propionate degradation by cocultures and tricultures with different methanogens. To test the abilities of different methanogens to act as the syntrophic partner organism in the syntrophic degradation of propionate, 10 ml of a fumarate-grown culture of MPOB was added to 40 ml of a hydrogen-grown culture of the methanogens. The gas phase was changed to N_2-CO_2, and propionate (15 mM) was added. The cocultures of MPOB with *Methanospirillum hungatei* (Fig. 1A) and *Methanobacterium formicicum* (Fig. 1B) degraded propionate to acetate and methane. The propionate degradation rates were about 0.8 mM/day and 0.4 mM/day, respectively. The methane yield was somewhat lower than expected because some fumarate (2 to 3 mM) was still present in the cocultures. The cocultures of MPOB with *Methanospirillum hungatei* and *Methanobacterium formicicum* could be subcultured by transfer to fresh media with propionate as the sole substrate. Propionate degradation was not observed in the cocultures of MPOB with *Methanobrevibacter arboriphilus* DSM 1125, *Methanobrevibacter arboriphilus* DSM 744, or *Methanothrix soehngenii* (results not shown). These incubations were continued for more than two months, but even after that period of time no propionate degradation had occurred. To test whether the distance between the MPOB and the H_2-consuming methanogen is of importance, bacteria were immobilized. After the addition of $FeCl_2$ (final concentration, 2 mM), the sulfide which was present in the media precipitated as FeS. During precipitation anaerobic bacteria coprecipitated, leading to high cell densities within the precipitates (26). Also, under these conditions propionate was degraded only in cocultures with *Methanospirillum hungatei* and *Methanobacterium formicicum*. The degradation rates were almost the same as those observed in the suspended cocultures (data not shown).

The effect of the additional presence of an acetate-degrading methanogen is shown in Fig. 2. The methanogenic triculture was constructed by mixing 20 ml of *Methanothrix soehngenii* culture, 20 ml of *Methanospirillum hungatei* culture, and 10 ml of MPOB culture. The biculture was constructed as described above. In the triculture 21 mM propionate was de-

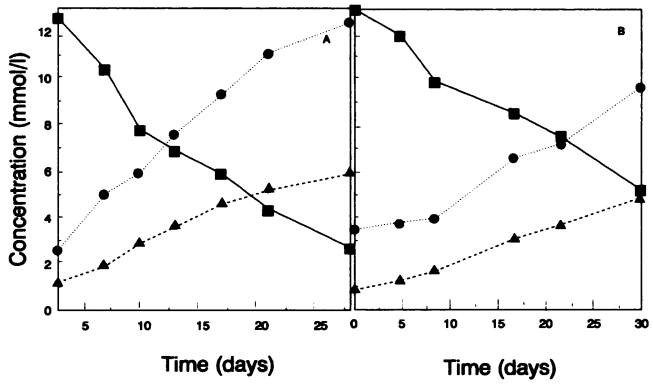

FIG. 1. Propionate (■) degradation and acetate (●) and methane (▲) production by MPOB in coculture with *Methanospirillum hungatei* DSM 864 (A) and *Methanobacterium formicicum* DSM 1535 (B).

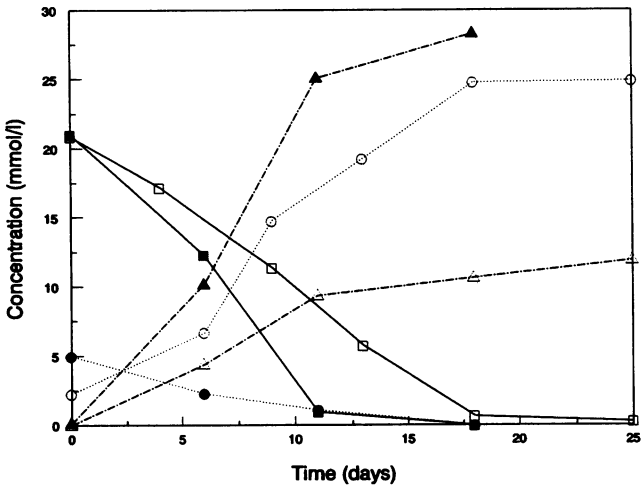

FIG. 2. Propionate (■, □) degradation and acetate (●, ○) and methane (▲, △) production by MPOB in triculture with *Methanospirillum hungatei* and *Methanothrix soehngenii* (closed symbols) and in biculture with *Methanospirillum hungatei* (open symbols).

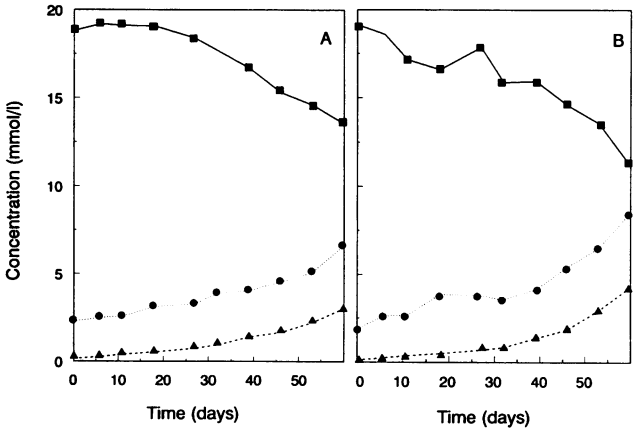

FIG. 3. Propionate (■) degradation and acetate (●) and methane (▲) production by tricultures of MPOB, *Methanobrevibacter arboriphilus* DSM 1125, and *Desulfovibrio* strain G11 (A) and MPOB, *Methanobrevibacter arboriphilus* DSM 1125, and the homoacetogenic bacterium EE121 (B).

graded and 28.3 mmol of methane per liter was produced within 18 days. The degradation rate was about 1.4 mM/day. Tricultures in which *Methanospirillum hungatei* was replaced by one of the *Methanobrevibacter* strains did not degrade propionate (results not shown).

Propionate degradation by tricultures consisting of MPOB, an H_2-utilizing methanogen, and a formate cleaving bacterium. *Desulfovibrio* strain G11 in the absence of sulfate (10) is able to cleave formate into H_2 and CO_2 (Table 1, equation 6), and the homoacetogenic bacterium EE121 (22) is able to convert formate partly into H_2 plus CO_2 and partly into acetate. Because the $\Delta G^{\circ\prime}$ of formate cleavage is around zero, a complete conversion of formate to H_2 is possible only when the H_2 is taken away by an H_2-utilizing methanogen. A possible role of formate in propionate oxidation can be demonstrated when propionate oxidation can be coupled to H_2 consumption by a methanogen in the presence of a bacterium able to cleave formate at a high rate. *Desulfovibrio* strain G11 in the absence of sulfate cleaved about 4 mM formate into 4 mM H_2, and the homoacetogen EE121 converted 4 mM formate into about 2 mmol of H_2 per liter in 7 days, the remainder presumably being converted to acetate. After the addition of *Methanobrevibacter arboriphilus* (DSM 1125) a much faster degradation of formate was observed, about 17 mM in 7 days. A triculture was constructed by mixing 30 ml of a formate-pregrown culture of *Desulfovibrio* strain G11 or the homoacetogen EE121, 10 ml of *Methanobrevibacter arboriphilus* DSM1125 culture, and 10 ml of MPOB culture. Figure 3 shows that propionate was degraded in the tricultures, whereas in the cocultures with either the homoacetogen EE121, *Desulfovibrio* strain G11, or *Methanobrevibacter arboriphilus* alone no degradation of propionate was observed (results not shown). In the triculture with *Desulfovibrio* strain G11 the propionate concentration had decreased by about 6 mM in 2 months (Fig. 3A), which corresponded with a degradation rate of about 0.15 mM/day. In the triculture with the homoacetogen EE121 a somewhat faster propionate degradation was observed; the propionate concentration decreased by 8 mM in 2 months (Fig. 3B), corresponding to a rate of 0.23 mM/day.

Effects of H_2 and formate on propionate degradation by MPOB in the absence of methanogens. To study the effects of hydrogen and formate on propionate degradation in the absence of methanogens, 162 kPa of H_2-CO_2 or 18 mM formate was added to MPOB growing on fumarate plus propionate. Figure 4A shows that propionate degradation was totally inhibited by both H_2 and formate and that acetate was even converted back to propionate. Fumarate conversion was not affected either in the culture with H_2 or in the culture with formate (Fig. 4B). As described previously, the ratio of fumarate degraded to succinate produced was almost 1:1 in the presence of formate or hydrogen, but in the absence of these electron donors the ratio was about 7:6 (27).

DISCUSSION

Thermodynamically both hydrogen and formate may be involved in the interspecies electron flow in syntrophic methanogenic cultures. The MPOB degraded propionate in coculture with methanogens which were able to use both hydrogen and formate. However, in cocultures with methanogens which are able to use only hydrogen, propionate was not degraded, unless bacteria which are able to convert formate into H_2 plus CO_2 were present. These findings indicate clearly that low formate and hydrogen concentrations are required for syntrophic propionate oxidation by MPOB, and they give support to the hypothesis that besides hydrogen transfer formate transfer is an important process as well (4, 30). Our data do not allow the quantification of the relative importance of the two processes. Boone et al. (4) calculated that in a syntrophic butyrate-degrading culture the formate transfer rate was 98-fold higher than the rate of hydrogen transfer.

The propionate degradation rate in the coculture with *Methanobacterium formicicum* was lower than that in the coculture with *Methanospirillum hungatei*, i.e., the rates were 0.8 and 0.4 mM/day, respectively. The K_m value of the formate dehydrogenase of *Methanobacterium formicicum* is 0.58 mM, and that of the enzyme of *Methanospirillum hungatei* is 0.22 mM; the formate threshold values of these methanogens are 26 and 15 μM, respectively (23). In contrast, the K_m values of the hydrogenases of these two methanogens are about the same, 6 and 5 μM, respectively, and the threshold values for hydrogen are 16.6 and 17.8 nM, respectively. In syntrophic degradation

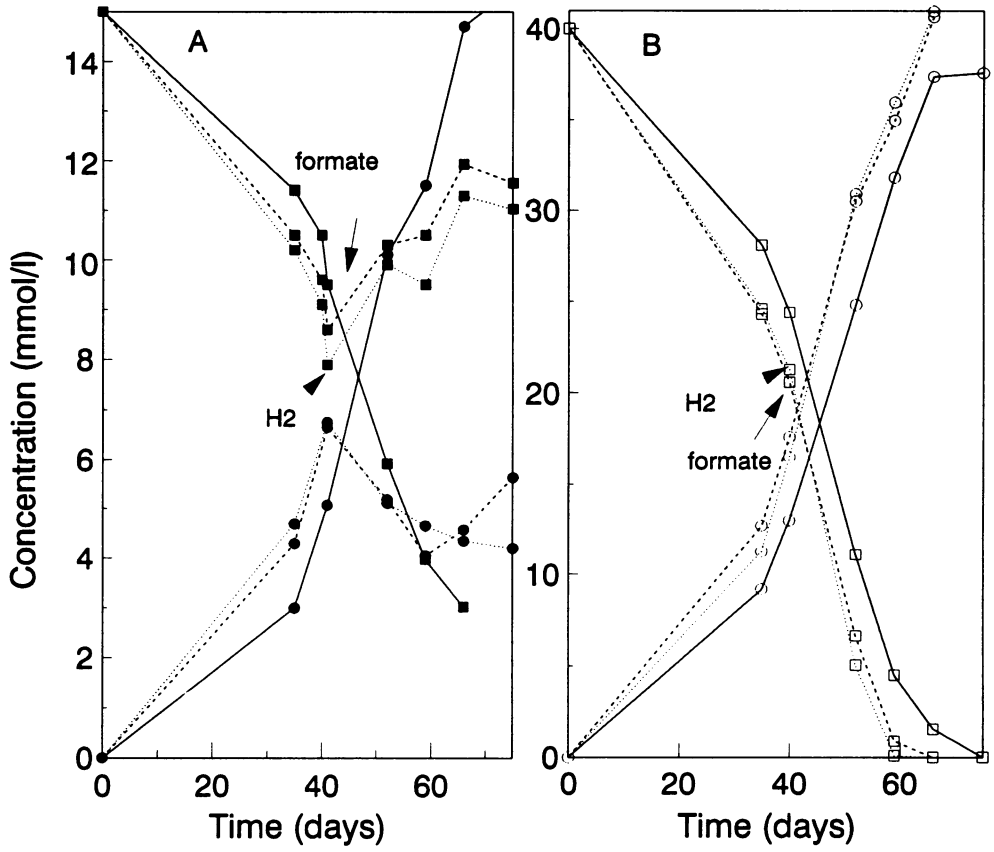

FIG. 4. Effect of H_2 and formate on propionate degradation (A) and fumarate degradation (B) by MPOB growing on propionate plus fumarate. Symbols: ■, propionate; ●, acetate; □, fumarate; ○, succinate. Solid lines represent the experiment without H_2 and formate. Dotted lines and dashed lines represent the experiments in the presence of 162 kPa of H_2 and 20 mM formate, respectively. The addition of H_2 or formate is indicated by the arrows.

the rate of conversion is determined by the rate of diffusion of hydrogen and/or that of formate from the acetogenic bacterium to the methanogenic bacterium. Diffusion rates of H_2 and formate are determined by the distance between the bacteria, the diffusion coefficients of H_2 and formate, and the gradients of H_2 and formate between the bacteria (25). Therefore, the differences in the propionate degradation rates in the cocultures can be explained best when the propionate oxidation rates are mainly determined by the concentration of formate and not by the concentration of hydrogen. Propionate is oxidized to acetate via the methylmalonyl-coenzyme A pathway (12, 13, 17). The oxidation of succinate to fumarate coupled to bicarbonate reduction to formate ($\Delta G^{\circ\prime}$ of 84.7 kJ/mol) or coupled to proton reduction to hydrogen ($\Delta G^{\circ\prime}$ of 86.2 kJ/mol) is the most energetically difficult step of this pathway. MPOB is also able to reduce fumarate to succinate with hydrogen or formate as an electron donor (27), and therefore both hydrogen and formate severely inhibit the succinate oxidation step during syntrophic growth on propionate. This implies that both the formate concentration and the hydrogen concentration have to be kept low in order for propionate oxidation to proceed. The observation that propionate can be degraded in tricultures in which a bacterium able to convert formate into hydrogen is present strongly suggests that hydrogen transfer can occur provided that low formate concentrations are maintained. Inhibition by formate might also explain why the shortening of the distance between the MPOB and *Methanobrevibacter arboriphilus* by coprecipitation with FeS was without effect and also why in tricultures consisting of the H_2-consuming methanogen *Methanobrevibacter arboriphilus* and *Methanothrix soehngenii* propionate degradation did not occur despite the fact that both the hydrogen concentration and the acetate concentration were kept low. At present it is not yet clear how formate is formed by MPOB. During propionate oxidation reducing equivalents are formed in three steps: succinate oxidation to fumarate, malate oxidation to oxaloacetate, and pyruvate oxidation to acetyl-coenzyme A. Cell extracts of MPOB contained hydrogenase and formate dehydrogenase activity (21), indicating that the bacterium is able to form both hydrogen and formate. The MPOB was also able to interconvert H_2 and formate. However, hydrogen was formed only when the formate concentration was above 0.9 mM, and formate formation occurred only when the H_2 partial pressure was above 80 kPa (unpublished data). During syntrophic growth on propionate, H_2 and formate concentrations were much lower, about 16 Pa and <0.1 mM, respectively. Formate can also be formed when pyruvate oxidation is catalyzed by a pyruvate formate lyase rather than by a ferredoxin-dependent pyruvate dehydrogenase (16, 29). Thus far we were unable to detect pyruvate formate lyase activity in cell extracts of MPOB.

It can be calculated that propionate oxidation cannot be driven by low acetate concentrations alone. Therefore, it was not surprising that cocultures of MPOB and *Methanothrix*

soehngenii did not degrade propionate. However, propionate was degraded much faster in tricultures consisting of MPOB, *Methanospirillum hungatei*, and *Methanothrix soehngenii* than in bicultures from which this aceticlastic methanogen (*Methanothrix soehngenii*) was absent. The propionate degradation rates were 1.4 and 0.8 mM/day, respectively. The lowest acetate concentration that can be reached by *Methanothrix soehngenii* is about 10 μM (15). Such low acetate concentrations are favorable for the syntrophic oxidation of propionate. It can be calculated that the $\Delta G^{\circ\prime}$ value of propionate oxidation is about 17 kJ lower when the acetate concentration is 10 μM than the $\Delta G^{\circ\prime}$ value when the acetate concentration is 10 mM. The beneficial effect of low acetate concentrations on syntrophic degradation of fatty acids has been reported before (1, 2, 27). In addition, syntrophic conversion of acetone plus CO_2 to 2 acetate molecules ($\Delta G^{\circ\prime}$ of −31.0 kJ/mol) is completely driven by interspecies acetate transfer (20).

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