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Methanogenic degradation of acetone by an enrichment culture

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Abstract. An anaerobic enrichment culture degraded 1 mol of acetone to 2 mol of methane and 1 mol of carbon dioxide. Two microorganisms were involved in this process, a filament-forming rod similar to *Methanothrix* sp. and an unknown rod with round to slightly pointed ends. Both organisms formed aggregates up to 300 µm in diameter. No fluorescing bacteria were observed indicating that hydrogen or formate-utilizing methanogens are not involved in this process. Acetate was utilized in this culture by the *Methanothrix* sp. Inhibition of methanogenesis by bromoethanesulfonic acid or acetylene decreased the acetone degradation rate drastically and led to the formation of 2 mol acetate per mol of acetone. Streptomycin completely inhibited acetone degradation, and neither acetate nor methane was formed. $^{14}CO_2$ was incorporated exclusively into the C-1 atom of acetate indicating that acetone is degraded via carboxylation to an acetoacetate residue. It is concluded that acetone is degraded by a coculture of an eubacterium and an acetate-utilizing methanogen and that acetate is the only intermediate transferred between both. The energetical problems of the eubacterium converting acetone to acetate are discussed.

Key words: Acetone – Acetate – *Methanothrix* sp. – Methanogenesis – Interspecies hydrogen transfer – Interspecies acetate transfer

Acetone is formed as a metabolic end product of various bacteria such as *Bacillus macerans* (Schardinger 1905; Northrop et al. 1919) *Clostridium acetobutylicum* (Davies and Stephenson 1941) and methanogens (Widdel 1986). It is also excreted by diabetic mammalians (Stryer 1981), and as a solvent it is released into nature by man.

Aerobic degradation of methyl ketones was first observed with hydrocarbon-utilizing bacteria (Lukins and Foster 1963). Acetone is degraded by bacteria (Taylor et al. 1980) and mammalian liver cells (for review, see Landau and Brunengraber 1987) via oxygenase-dependent hydroxylation to acetol. Methanogenic degradation of acetone was first observed by Mazé (1915) and confirmed in two later publications (Symons and Buswell 1933; Wikén 1940). It was further observed that anaerobic enrichment with acetone in the presence of sulfate selects for *Desulfococcus multivorans* (Widdel 1987). In the present study we describe methanogenic degradation of acetone in an enrichment culture and provide evidence that acetone is degraded in this system by interspecies acetate transfer.

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Materials and methods

Source of microorganisms

The culture WoAct was enriched from anoxic sediment of a polluted freshwater creek near Konstanz, FRG.

Media and growth conditions

All procedures for cultivation were as previously described (Widdel and Pfennig 1981). The freshwater mineral medium (Schink and Pfennig 1982) for enrichment and further cultivation was carbonate-buffered (30 mM) and sulfide-reduced (1.0 mM), and contained 1 ml of trace element solution SL 10 and 1 ml of selenite-tungstate solution (Widdel et al. 1983) per liter as well as 0.5 ml · l^{-1} vitamin solution (Pfennig 1978). Resazurin (4 mg · l^{-1}) was added as redox indicator. The pH was adjusted to 7.2–7.4. Acetone was added either undiluted or as a 1.0 M solution in sterile water. 60 or 120 ml serum bottles were filled half with medium under N_2/CO_2 (90%/10%) and sealed with butyl rubber stoppers. Cultures were incubated at 28°C.

Preparation of cell suspensions

300 ml of freshwater medium containing 300 µl acetone (13.6 mM) in a 500 ml bottle was inoculated with 50 ml of WoAct culture pregrown with 50 µl acetone. After 90 days of incubation, the culture was fed with another 300 µl acetone and further incubated for 1 month. The culture was transferred anoxically into 120 ml serum bottles and centrifuged for 20 to 30 min at 4°C in a Sorvall RC-5 centrifuge (GSA rotor, 2,100 × *g*). The pellet collected from 300 ml medium was washed once with freshwater mineral medium and resuspended in 50 to 75 ml freshwater medium. This cell suspension was distributed anoxically to 2 or 3 60 ml serum bottles (approx. 25 ml per bottle) under N_2/CO_2 (90%/10%) gas mixture.

Inhibition experiments

Stock solutions of streptomycin sulfate (10 mg · ml^{-1}, Sigma, St. Louis, MO, USA), penicillin G, K-salt (10 mg · ml^{-1}, Serva, Heidelberg, FRG) and sodium bromoethane-

sulfonate (BES, 1 mol · l^{-1}, Serva, Heidelberg, FRG) were prepared in boiled water with resazurine (4 mg · l^{-1}), reduced with sodium dithionite (approx. 1 mg per 25 ml) and filter-sterilized. Acetylene was obtained from Messer-Griesheim (Düsseldorf, FRG) and injected directly into the culture bottles. Its concentration in the liquid phase was calculated after Flett et al. (1976).

Chemical analyzes

Acetate and acetone were assayed by gas chromatography in a Carlo Erba Vega 6000 gas chromatograph with flame ionization detector and a glass column (2 m × 2 mm) packed with 60/80 Carbopak C/0.3% Carbowax 20 M/0.1% H_3PO_4 (Supelco, Inc.), injector and detector temperature 140°C, column temperature 120°C, carrier gas nitrogen, 45 ml · min^{-1}. Samples were acidified prior to injection with formic acid from 10 M stock solution to 0.5 M final concentration. Chromatograms were recorded with a Spectraphysics SP 4290 integrator.

Methane and carbon dioxide were quantified with a similar gas chromatograph. For methane, a 2.0 m/2 mm column with a molecular sieve (5 Å, 18–50 mesh, Merck, Darmstadt, FRG) was used, column temperature 120°C, detector and injector temperature 140°C. Carbon dioxide was measured with a 2.0 m/2 mm column packed with Carbosieve B (Supelco Inc.), oven temperature 150°C, detector and injector temperature 170°C after converting to methane with a methanizer. In both cases, nitrogen was the carrier gas with a flow rate of 40 to 50 ml · min^{-1}. Chromatograms were evaluated with a BBC recorder.

Tracer experiments with $^{14}CO_2$

5 µl of $Na_2{}^{14}CO_3$ solution (36.3 mM; 55 mCi · $mmol^{-1}$) obtained from Amersham (Braunschweig, FRG) was added to 25 ml of concentrated cell suspension. For determination of the specific radioactivity of CO_2, 1–2 ml of the headspace of the culture after 26 to 30 days was transferred into a 8.5 ml serum bottle gassed with nitrogen. After determination of the CO_2 content, 1 ml 1 M KOH was added. The solution was stirred for 1 h and its radioactivity was determined. Acetate was prepared from culture broth as follows: 500 µl of a culture sample was acidified with 500 µl 3 M $HClO_4$, stirred for 2 min to remove CO_2, and neutralized with 250 µl 6 M KOH. After freezing to −20°C, $KClO_4$ was removed by centrifugation for 2 min at room temperature in a Hettich microliter centrifuge (13,000 × *g*). The supernatant was run over a Dowex 1X8 column (formate form, 100–200 mesh, 15 × 0.4 cm) and eluted with 50 mM formic acid. The eluate was concentrated in a vacuum evaporator after alkalinization.

Schmidt degradation of acetate was carried out after Simon and Floss (1967) using a special degradation apparatus (Fuchs et al. 1980).

Radioactivity was determined with a Beckmann LS 7500 scintillation counter with 20 to 50 µl samples in 5 ml Aqualuma (J.T. Baker Chemicals, Deventer, Netherland).

Results

Enrichment, microscopy

50 ml-enrichment cultures with 10 mM acetone as sole carbon and energy source were inoculated with 5 ml sewage sludge or sediment samples from freshwater and marine sources. Methane formation started after 4 to 8 weeks in 2 of 6 cultures, one freshwater and one marine one, and ceazed again after another 2 weeks. Transfers were made with 5 ml enrichment culture including part of the sediment to subsequent 50 ml-subcultures. After 3–5 transfers, the culture enriched from freshwater creek sediment (WoAct) proved to be the most active one and was used for the present studies after more than 20 transfers over nearly 4 years. The culture consisted mainly of two different bacteria. The first one was a rod, 1 µm in diameter forming long filaments (up to several hundred micrometers) which disintegrate into single cells (approx. 2.5 µm in length), especially in elder cultures. Elder cells often contained highly refractive inclusions, probably gas bubbles or gas vacuoles. The other bacterium was a rod as well, 1 µm × 1.5 µm with round to slightly pointed ends. Both bacteria formed closely associated microcolonies, 50–300 µm in diameter. Epifluorescence microscopy at 420 nm wavelength gave no indication of fluorescing cells. All efforts to isolate an acetone-degrading bacterium from this culture failed so far, no matter whether dilutions were made in agar shakes or in liquid media.

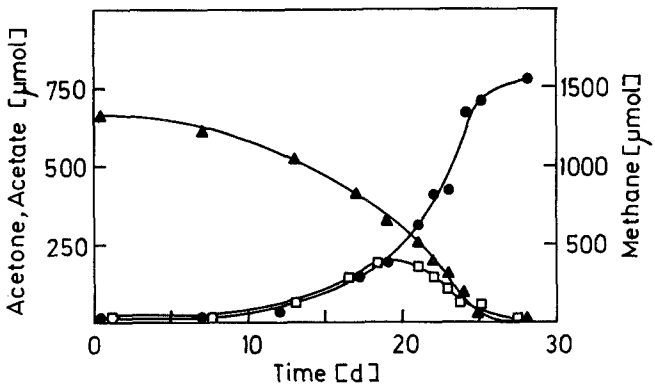

Fig. 1. Conversion of acetone (10 mM) to acetate and methane by enrichment culture WoAct. (▲) Acetone, (□) acetate, (●) methane

Optimization of growth conditions

After inoculation of 50 ml medium containing 10 mM acetone with 5 ml of a culture pregrown under the same conditions, acetone was completely degraded to methane and CO_2 within 28 to 35 days (Fig. 1). CO_2 formation was not determined because of the high CO_2 background in the carbonate-buffered medium used. The stoichiometric conversion of 1 mol acetone to 2 mol methane was confirmed in more than twelve similar growth experiments. Sodium dithionite enhanced acetone degradation (measured as methane formation) at 4 to 10 mg · l^{-1}. Concentrations ≥ 50 mg · l^{-1} had an inhibitory effect. Sodium chloride (≥ 64 mM) and sulfide (> 2 mM) also inhibited methane formation. From the methane formation rates (Fig. 1) a doubling time of 2.8 to 3.5 days was calculated for the methanogenic bacterium in the culture.

Acetate was formed as an intermediate during acetone degradation (Fig. 1). The highest concentration of acetate found in a growing culture was 2.5–2.8 mM. If acetate instead of acetone was added as substrate only the filamentous bacterium grew and formed 1 mol methane from 1 mol acetate. The doubling time was the same as with

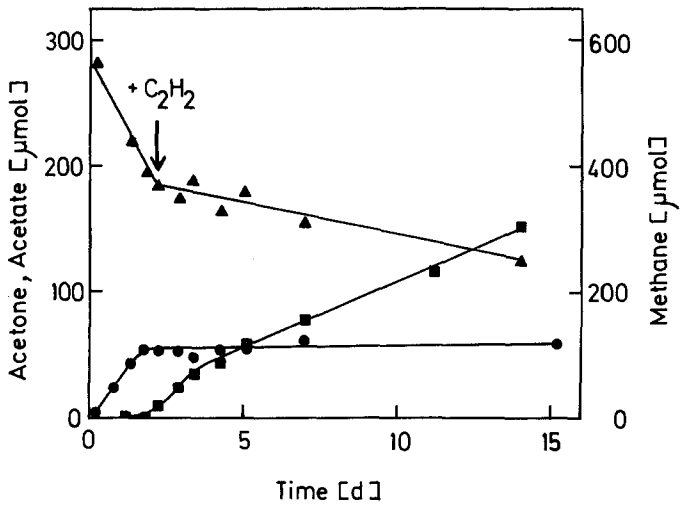

Fig. 2. Inhibition of methane formation in dense suspensions of culture WoAct by acetylene (42 µM). The culture volume was 25 ml. Acetone (10 mM) was initially degraded at a rate of 2 µmol · h^{-1}. (▲) Acetone, (■) acetate, (●) methane

acetone as substrate, but the lag-phase was about 12 days shorter (not shown).

Influence of inhibitors on acetone degradation

Acetylene (16 µM) inhibited methane formation in a freshly inoculated WoAct culture completely. In the inhibited culture acetone was converted to acetate only, but at a very low rate: only 20 to 50% of the initial acetone was converted to acetate in about 100 days. Bromoethanesulfonate (BES) at a concentration of 200 µM did not inhibit methane formation completely. Treatment with either acetylene or BES did not lead to a selective enrichment of one single type of bacteria.

Because of the extremely slow growth of the inhibited cultures, concentrated cell suspensions were used to elucidate the action of inhibitors. Addition of acetylene (42 µM) to a cell suspension immediately stopped methane formation completely and decreased the acetone degradation rate by 88%, from originally 80 nmol · h^{-1} · ml^{-1} to 9.2 nmol · h^{-1} · ml^{-1} (Fig. 2). Approximately two moles of acetate were formed per mol of acetone degraded (19.6 nmol · h^{-1} · ml^{-1}, Fig. 2). BES had the same effect (Fig. 3c). A concentration of 20 mM was used to ensure that methanogenesis was completely inhibited. If acetate was provided together with acetone, acetate was used up first before acetone was degraded (Fig. 4a). If this culture was inhibited with BES neither acetate nor acetone was degraded (Fig. 4c).

The influence of inhibitors of eubacteria was also tested. Penicillin G had no effect at all at a concentration of 100 µg · ml^{-1}. Streptomycin at the same concentration did not affect methane formation from acetate (Fig. 4b) but completely prevented acetone conversion to acetate (Figs. 3b, 4b).

Incorporation of $^{14}CO_2$ into acetate

To elucidate the initial step of acetone degradation, dense cell suspensions were incubated with radioactive bi-

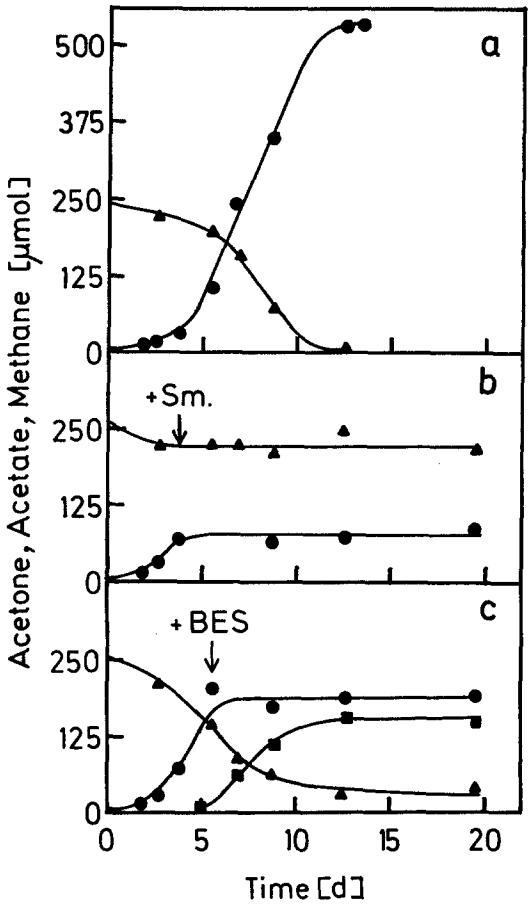

Fig. 3a–c. Influence of inhibitors on dense suspensions of culture WoAct with acetone (10 mM) as substrate. Inhibitors were added after the onset of methane formation from acetone. **a** Untreated control; **b** streptomycin (*Sm.*, 100 µg · ml^{-1}) added after 3.5 days; **c** bromoethanesulfonate (*BES*, 20 mM) added after 5.2 days. (▲) Acetone, (■) acetate, (●) methane

carbonate. As soon as degradation of acetone began, methane formation was inhibited either with BES or with acytelene as described above. After 26 to 30 days of incubation on a rotary shaker (160 rpm), the specific radioactivities of CO_2 and of the acetate formed were determined. The specific radioactivity of $^{14}CO_2$ over ^{14}C-acetate turned out to be nearly exactly 2:1 (Table 1). Schmidt degradation of the labeled acetate showed that only the C-1-carbon atom was labeled (Table 2).

Discussion

In the presence of molecular oxygen, acetone is hydroxylated by oxygenases to acetol, and further oxidized via methylglyoxal and pyruvate (Taylor et al. 1980). In the absence of oxygen, such a reaction is not possible, and anaerobic acetone degradation has to take a basically different route, therefore.

In the present study, acetone degradation by a methanogenic enrichment culture was found to proceed according to the following equation (calculation of free reaction energies after Thauer et al. 1977):

$$CH_3COCH_3 + H_2O \rightarrow 2\,CH_4 + CO_2$$

$$\Delta G^{\circ\prime} = -89.2\ \text{kJ} \cdot \text{mol}^{-1}. \quad (1)$$

Table 1. Incorporation of $^{14}CO_2$ into acetate during acetone degradation. 25 to 30 ml cell suspension was incubated on a rotary shaker (160 rpm) with 10 mM acetone and 5 µl of a labeled sodium carbonate solution (36.3 mM, 55 mCi · mmol^{-1}) in the presence of BES (20 mM) or acetylene (42 µM). After 30 days, the specific radioactivities of carbon dioxide and acetate were determined. No acetate was detectable at the beginning of the experiment. Results of three representative experiments are shown

Amount of CO_2 in 1 ml KOH (µmol)	Radioactivity of CO_2 in 1 ml KOH (dpm)	Specific radioactivity of CO_2 in the culture (dpm/µmol)	Acetate conc. in the preparation (µmol/ml)	Radioactivity of the acetate preparation (dpm/ml)	Specific radioactivity of acetate (dpm/µmol)	Ratio $\frac{^{14}CO_2}{^{14}C\text{-acetate}}$
7.1	70,100	9,873	10.2	47,850	4,691	2.10
7.7	88,658	11,514	4.6	25,220	5,483	2.10
17.8	198,703	11,163	7.3	42,983	5,888	1.90
Mean		10,850			5,354	2.03

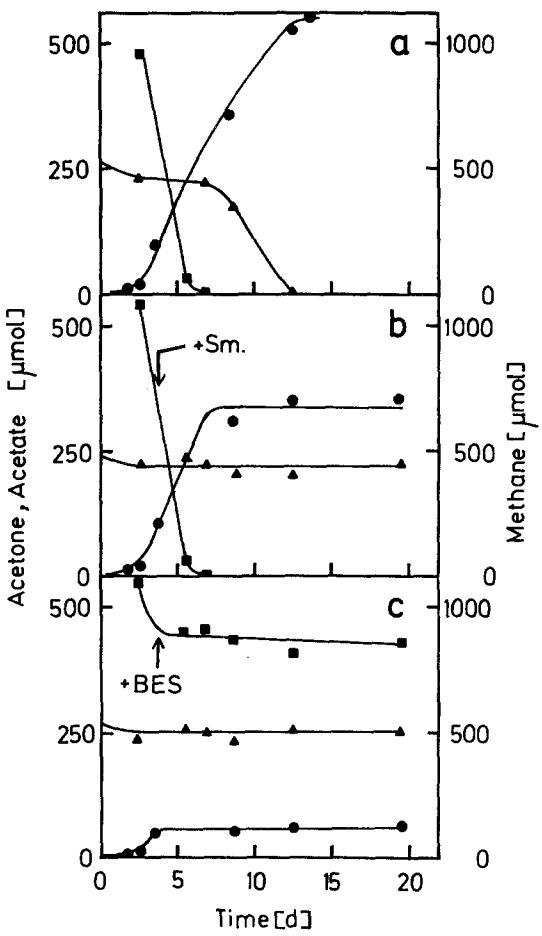

Fig. 4a–c. Influence of inhibitors on dense cell suspensions of culture WoAct with acetate (20 mM) and acetone (10 mM) as substrates. Acetate was added after the onset of methane formation (2.5 days). **a** Untreated control; **b** streptomycin (*Sm.*, 100 µg/ml) added after 3.5 days; **c** bromoethanesulfonate (*BES*, 20 mM) added after 3.5 days. (▲) Acetone, (■) acetate, (●) methane

The same stoichiometry was published earlier on the basis of measurements with sludges and enrichment cultures (Mazé 1915; Symons and Buswell 1933; Wikén 1940). Basically three pathways are possible by which acetone could be converted to methane and CO_2. i) a methanogenic bacterium could use acetone directly [Eq. (1)]; ii) a

Table 2. Schmidt degradation of ^{14}C-acetate prepared from WoAct culture broth incubated in the presence of BES as described in the legend to Table 1. For control, a 1-^{14}C-acetate solution with 4.1 · 10^6 dpm/ml and a 2-^{14}C-acetate solution with 3.4 · 10^6 dpm/ml were treated in the same manner as the culture samples. Preparation 1: 800 µl containing 4.0 mM acetate, radioactivity: 23,000 dpm/ml; Preparation 2: 800 µl containing 6.9 mM acetate, radioactivity: 40,750 dpm/ml

Sample	Radioactivity (dpm) in		
	Acetate (total)	Carbon atom	
		C-1	C-2
1-^{14}C-acetate	1.62 · 10^6	1.64 · 10^6	0.01 · 10^6
2-^{14}C-acetate	1.34 · 10^6	0.003 · 10^6	0.88 · 10^6
Prep. 1	18,400	19,418	272
Prep. 2	32,600	32,670	720

fermenting bacterium could produce either carbon dioxide and hydrogen [Eq. (2)] or acetate, carbon dioxide and hydrogen [Eq. (3)] in syntrophic cooperation with hydrogen-scavenging and acetate-utilizing methanogens; iii) a fermenting bacterium could produce acetate only in coculture with an acetate-utilizing methanogen [Eq. (4)].

$$CH_3COCH_3 + 5\,H_2O \rightarrow 3\,CO_2 + 8\,H_2$$
$$\Delta G^{\circ\prime} = +\,189.0\ kJ \cdot mol^{-1} \quad (2)$$

$$CH_3COCH_3 + 3\,H_2O \rightarrow CH_3COO^- + H^+ + CO_2 + 4\,H_2$$
$$\Delta G^{\circ\prime} = +\,77.4\ kJ \cdot mol^{-1} \quad (3)$$

$$CH_3COCH_3 + CO_2 + H_2O \rightarrow 2\,CH_3COO^- + 2\,H^+$$
$$\Delta G^{\circ\prime} = -\,34.2\ kJ \cdot mol^{-1}. \quad (4)$$

Our results prove that acetone degradation in our enrichment culture follows Eq. (4). Microscopic examination showed that two types of bacteria forming stable flocs are involved in acetone transformation to methane. The filamentous bacterium strongly resembles *Methanothrix* sp. All strains of this species isolated so far use only acetate as energy source (Zehnder et al. 1980; Fathepure 1983; Patel 1984, Zinder et al. 1987). Addition of acetate to our enrichment culture led to a selective growth of this bacterium. Furthermore, the lack of fluorescing methanogens in our culture indicates that molecular hydrogen, methanol, or

formate are not important intermediates in acetone degradation (Dolfing and Mulder 1985). A pathway consistent with Eqs. (2) and (3) can be ruled out, therefore.

These observations led to the hypothesis that in our culture an eubacterium degrades acetone to acetate in cooperation with an acetate-utilizing methanogen. This hypothesis was confirmed by inhibition experiments. Streptomycin inhibited acetone conversion to acetate and did not affect acetate degradation, whereas the latter was completely inhibited by bromoethanesulfonate and acetylene. However, the latter two also impeded acetone degradation to a high extent (Figs. 2, 4c). Direct inhibition of the acetone-degrading bacterium by these agents cannot be ruled out at the moment, however, appears to be rather improbable in view of their high specifity for methanogenic bacteria (Gunsalus et al. 1978; Sprott et al. 1982). Streptomycin, on the other hand, does not inhibit methanogenic bacteria (Pecher and Böck 1981). An indirect effect via acetate accumulation seems more plausible.

This hypothesis is supported by the observation that acetone degradation was completely inhibited by acetate at high concentrations (20 mM, Fig. 4a, c). Both results indicate that successful acetone degradation depends on an efficient removal of acetate. The observed tendency of the two partners in our culture to form stable, densely intricated flocs may be a suited means to ensure an efficient acetate transfer from the producer to the consumer. Floc formation of this kind has so far been observed with anaerobic communities depending on interspecies hydrogen transfer (Dolfing et al. 1985). Analogous to this, one could characterize the apparent mutual dependence of the two partners in methanogenic acetone degradation as "interspecies acetate transfer". Acetate transfer in complex methanogenic degradation chains has usually been regarded to be only of minor importance compared to interspecies hydrogen transfer (Bryant et al. 1967), however, may play a key role in some special cases, e.g. isovalerate degradation (Stieb and Schink 1986). Conversion of acetone to methane and CO_2 represents the first case in which acetate is the only intermediate transferred between a fermenting eubacterium and a methanogen.

Labeling experiments with $^{14}CO_2$ indicated that the first reaction in acetone degradation is a carboxylation, probably to acetoacetate. This reaction would be the reversal of that employed in acetone formation by clostridia which was found to be irreversible under physiological conditions (Westheimer 1963). Our labeling experiments demonstrated that labeled CO_2 is only incorporated into the C-1 atom of acetate, and that the specific radioactivity of the two acetate residues formed is exactly half of that of the CO_2 provided. These experiments again support the hypothesis that methanogenic acetone degradation proceeds via an intermediate formation of two acetate residues according to Eq. (4). The same pathway is probably being used by other anaerobic acetone degraders, e.g. *Desulfococcus multivorans* (Widdel 1987) or the nitrate-reducing *Thiosphaera pantotropha* (G. Kuenen unpublished work).

The energy metabolism of the acetone-fermenting eubacterium in our enrichment culture deserves some consideration. The total free energy change under standard conditions [-34.2 kJ · mol^{-1}, Eq. (4)] is too small to allow synthesis of 1 mol ATP per mol acetone by substrate level phosphorylation since irreversible ATP synthesis under physiological conditions requires at least 60–70 kJ · mol^{-1} (Thauer et al. 1977). The free energy change of the overall reaction under the conditions of the experiment ($\Delta G' = -70$ kJ · mol^{-1} for 10 mM acetone, 100 μM acetate, 30 mM CO_2) is considerably more negative than under standard conditions [cf. Eq. (4)], which explains why acetone degradation depends on acetate consumption by the methanogenic partner. The initial carboxylation of acetone to acetoacetate is an endergonic reaction [$\Delta G^{\circ\prime} = +17.1$ kJ · mol^{-1}, Eq. (5)] which requires to be driven by metabolic energy:

$$CH_3COCH_3 + CO_2 \rightarrow CH_3COCH_2COO^- + H^+ \quad \Delta G^{\circ\prime} = +17.1 \text{ kJ} \cdot \text{mol}^{-1}. \qquad (5)$$

Since not the whole equivalent of one ATP as with many biotin-dependent carboxylation reactions (Stryer 1981) can be spent in this step, one has to think of a possible membrane-bound mechanism which couples the carboxylation reaction with an ion transport across the membrane. Such a process which requires only fractions of an ATP have been studied in the recent past (Dimroth 1982; Hilpert et al. 1984) and a similar mechanism was assumed to operate in methanogenic isovalerate degradation (Stieb and Schink 1986). Our further studies will concentrate on isolating the acetone-fermenting eubacterium and elucidate the pathway of its energy metabolism.

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