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Acetate Production by Methanogenic Bacteria

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***Methanosarcina barkeri* MS and 227 and *Methanosarcina mazei* S-6 produced acetate when grown on H_2-CO_2, methanol, or trimethylamine. Marked differences in acetate production by the two bacterial species were found, even though methane and cell yields were nearly the same. *M. barkeri* produced 30 to 75 μmol of acetate per mmol of CH_4 formed, but *M. mazei* produced only 8 to 9 μmol of acetate per mmol of CH_4.**

Acetate is one of the most important intermediates produced during degradation of organic matter in anaerobic environments (3, 19, 20). During fermentation of organic compounds, H_2, CO_2, acetate, and other volatile fatty acids, such as propionate and butyrate, are formed. Butyrate and propionate may be either degraded to acetate, H_2, and CO_2 by obligate proton-reducing acetogens in the absence of sulfate or nitrate or oxidized completely to CO_2 and H_2O by sulfate-reducing or nitrate-reducing bacteria in the presence of these inorganic electron acceptors. Autotrophic acetogenic bacteria such as *Acetobacterium*, *Acetogenium*, *Eubacterium*, and *Clostridium* species (1, 10, 17, 18, 23) are capable of synthesizing acetate by CO_2 reduction with H_2 or by fermentation of organic compounds. Only a few studies on the relative quantitative significance of autotrophic acetogenesis in anaerobic ecosystems have been reported. Jones and Simon (7) and Lovly and Klug (12) showed that less than 10% of the acetate was derived from H_2-CO_2 in freshwater sediments. Prins and Lankhorst (15) reported that acetogenesis from H_2-CO_2 in the ceca of rodents was important only in the absence of methanogenesis.

Many *Methanosarcina* species metabolize acetate, H_2-CO_2, methanol, and methylamines to CH_4 and CO_2. In anaerobic environments such as digestors, in which the acetate concentration may be high, *Methanosarcina* species are probably important acetate degraders because they are able to convert acetate to methane at faster rates than other aceticlastic methanogens (6).

During a study of methanol kinetics, we observed that *Methanosarcina barkeri* produced extracellular acetate at millimolar concentrations when either H_2-CO_2 or methanol was the substrate. The aims of the present study were to quantify this activity and to determine if acetate production was a characteristic of other methanogenic bacteria.

MATERIALS AND METHODS

Organisms. *M. barkeri* 227 and MS, *Methanosarcina mazei* S-6, *Methanobacterium formicicum* HR, and *Methanobacterium thermoautotrophicum* were from our culture collection.

Culture media and conditions. Strictly anaerobic conditions were maintained throughout our studies. The anaerobic techniques used were essentially those described by Hungate (5) as modified by Bryant (2). We used alpha medium without Trypticase (BBL Microbiology Systems)-peptone and yeast extract for the experiments (24). All experiments were performed in 50-ml serum vials (20 ml of medium) closed with butyl rubber stoppers and aluminum crimps. The gas phase was N_2-CO_2 (7:3) pressurized to 170 kPa with H_2 when necessary. Trimethylamine and methanol were added

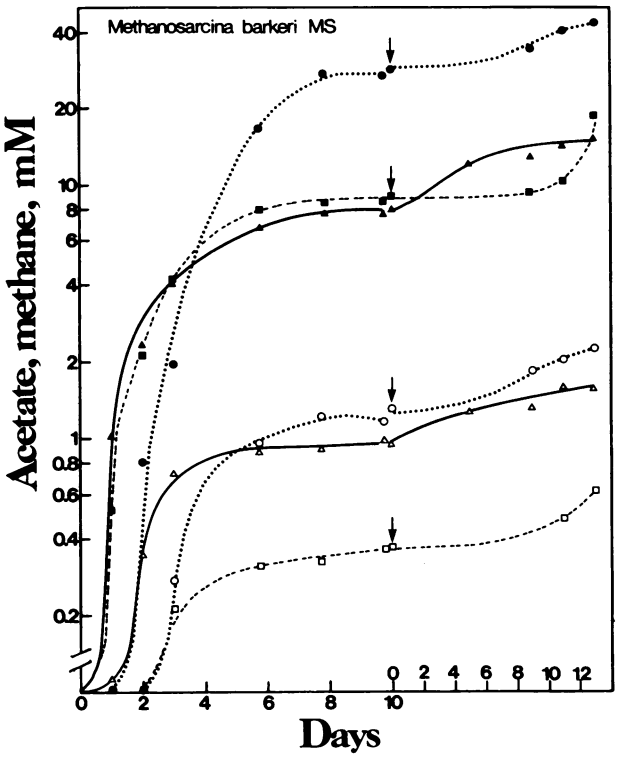

FIG. 1. Methane and acetate production by *M. barkeri* MS when grown on trimethylamine (● and ○), methanol (■ and □), or H_2-CO_2 (▲ and △). Open symbols, acetate production; closed symbols, methane production. Arrows indicate further substrate additions.

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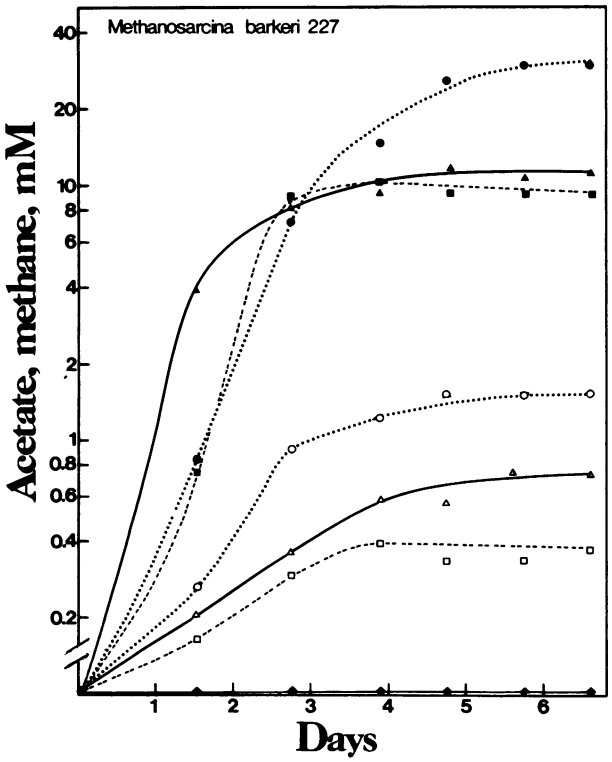

FIG. 2. Methane and acetate production by *M. barkeri* 227. ◆, Acetate and methane production when 2-bromomethanesulfonic acid was added to the cultures. Other symbols are explained in the legend to Fig. 1.

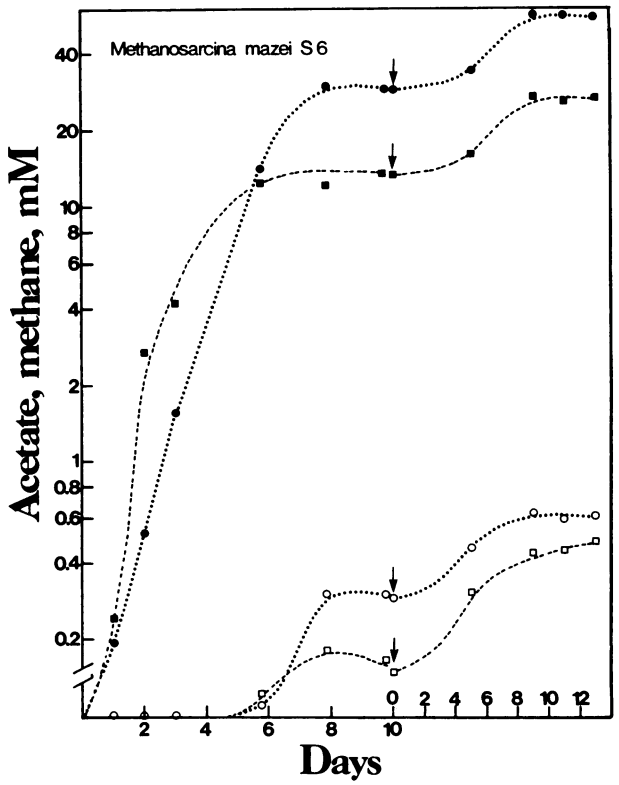

FIG. 3. Methane and acetate production by *M. mazei* S-6. Symbols are explained in the legend to Fig. 1.

to the vials from sterile stock solutions stored under N_2 to give a final concentration of 10 to 50 mM. All cultures were incubated at 37°C in a shaking incubator.

Isotope experiments. Sterile anaerobic solutions of $NaH^{14}CO_3$ (specific activity, 50 mCi per mmol; Amersham Corp.) were injected into vials inoculated with *M. barkeri* 227 and with either H_2 or trimethylamine as substrate. When stationary phase was reached, the cells were killed by acidifying the vials to pH 1 with H_3PO_4. Residual and produced labeled CO_2 and CH_4 were removed by flushing the vials with air. Less than 0.015% of the added counts remained in the solution in uninoculated controls. After sedimentation of the cells, 1 to 2 ml of supernatant fluid was removed and counted in Aquasol scintillation liquid with [^{14}C]acetate as the internal standard.

Cell yield. Cell dry weight was determined by filtering 50-ml culture samples through preweighed membrane filters (pore size, 0.22 μm; Millipore Corp.), washing the filters with HCl (0.01 N), and drying them to a constant weight at 98°C. The increase in weight was taken as the dry weight.

Analytic procedures. CH_4 and CO_2 were measured with a gas chromatograph equipped with a thermal conductivity detector (Aerograph; column, 3.65 m, aluminum packed with activated charcoal; oven temperature, 180°C; injector and detector temperature, 60°C; Varian). Acetate was measured by capillary gas chromatography (model 5890 chromatograph [column, 10 m by 0.53 mm], Hewlett-Packard Co.; Superox, 1.2 μm [Alltech Associates, Inc.]; oven temperature, 105°C; injector and detector [flame ionization] at 180°C). Samples were acidified to a pH of <2 with H_3PO_4 and centrifuged, and 0.5 μl was analyzed.

RESULTS

The two strains of *M. barkeri* and *M. mazei* produced acetate during metabolism of methanol, trimethylamine, or H_2-CO_2 (Fig. 1, 2, and 3). The amount of acetate produced did not vary significantly whether the bacteria were grown with or without yeast extract and Trypticase-peptone in the medium (data not shown). However, the growth rate, and hence the acetate production rate, was higher with the organic constituents in the medium. To avoid interferences from amino acid degradation or similar sources of acetate, we chose to grow the bacteria in a mineral medium.

Figures 1, 2, and 3 show that when grown on methanol or trimethylamine, the two *M. barkeri* strains produced two to three times more acetate than *M. mazei*. Since *M. mazei* uses H_2 only slowly (13), this substrate was not tested with this organism. When 2-bromoethanesulfonic acid, a specific inhibitor of methanogenesis, was added to cultures of *M. barkeri* 227, neither methane nor acetate production was observed in the vials, showing that acetate production was a function of the activity of this organism (Fig. 2). When growth terminated after 10 days, the vials containing *M. barkeri* MS and *M. mazei* S-6 were left in the incubator for another 10 days. During this period, no significant increase or decrease in methane or acetate concentrations occurred. After these 10 days, all vials were supplied with the same amount of substrate as had initially been added. The bacteria resumed growth and methane production after a lag period, and acetate concentrations increased until new levels were reached upon exhaustion of the substrate.

When ^{14}C-labeled $NaHCO_3$ was added to vials inoculated with *M. barkeri* 227 and containing trimethylamine or H_2-CO_2 as the substrate, 5.8 to 11.9% of the added tracer

TABLE 1. Conversion of $NaH^{14}CO_3$ to nonacid volatile products by *M. barkeri* 227

Substrate	$NaH^{14}CO_3$ added (kdpm)	Residual count (kdpm)[a]	Production (mM)[a] of:		
			Acetate (actual)[b]	Methane[b]	Acetate (theoretical)[c]
H_2-CO_2	43.85	2.55 (0.11)	1.27 (0.11)	16.76 (1.18)	1.86
TMA[d]	43.85	5.22 (0.62)	2.20 (0.61)	73.57 (7.77)	3.80

[a] Values in parentheses are standard deviations.
[b] Measured by gas chromatography.
[c] Calculated as (residual level × total CO_2)/(added label × 2).
[d] TMA, Trimethylamine.

remained in solution as a nonacid volatile label after removal of cells by centrifugation. The only short-chain fatty acid which was found upon gas chromatography analysis was acetate. Because the theoretical acetate production (Table 1) was greater than the measured acetate concentration, other cell products might have been labeled either directly during assimilatory activity or through isotope exchange reactions. Furthermore, water-soluble labeled compounds from dead cells might have contributed to the residual counts found in the medium.

When *M. formicicum* and *M. thermoautotrophicum* were grown on H_2-CO_2, the acetate concentration in the medium never rose above the detection limit of the gas chromatograph (20 μM) (data not shown).

Table 2 shows the relationship between acetate-methane production and acetate production per cell yield when the three organisms were grown on trimethylamine or methanol. When differences in yield (milligrams of cells per millimole of methane) were tested by the *t* test, no significant differences among the three bacteria were observed (confidence limit, 89.35%). There were no significant differences between the two strains of *M. barkeri* regarding acetate production as a function of methane production (confidence limit, 62.82%), while there was a significant difference between the two *M. barkeri* strains and *M. mazei* (confidence limit, 99.99%). The same pattern was observed when acetate production was measured as a function of cell yield.

DISCUSSION

The synthesis of acetate from H_2-CO_2 in enrichment cultures inoculated with mud and fermenting seaweed is generally associated with autotrophic acetogenic rather than methanogenic bacteria. Formation by these latter bacteria of extracellular compounds other than volatile acids was reported by Zehnder and Wuhrmann (26), who found that *Methanobrevibacter arboriphilus* formed significant quantities of amino acids, especially alanine, when it was grown on H_2-CO_2 in mineral medium. *M. barkeri* produced H_2 during metabolism of acetate, methanol, or trimethylamine (11). In mixed-culture methanogenic fermentations, *M. barkeri* may harbor several species of anaerobic bacteria inside the pockets and crevices of the large pseudosarcinal aggregates (28). These nonmethanogenic bacteria may metabolize dead-cell residues and extracellular products formed by the methanogens. In anaerobic acetate-metabolizing enrichments, as many as 10^8 nonmethanogenic bacteria per ml may occur (22). Nutritional characterization of several of these heterotrophs indicated that a variety of nutrients could be supplied by the *Methanosarcina* species present in the enrichment system in which acetate was the sole carbon and energy source.

Extracellular formation of low to trace concentrations of acetate by methanogenic bacteria was previously reported (4, 25). However, in the isotope exchange study of Eikmanns and Thauer (4), 0.7 mM acetate from CO_2 and CO was observed only in acetate-grown suspensions of *M. barkeri*. Acetate was the only substrate tested in this study. We calculated from the data of Zehnder and Brock (25) that *M. barkeri* and *Methanothrix soehngenii* produced acetate from CH_4 and CO_2 at a concentration of less than 0.005 to 0.014 mM. This concentration is too low to be an important source of the considerably higher concentrations of acetate reported in our current study.

In *M. thermoautotrophicum*, small amounts of labeled CO_2 were incorporated into acetate during pulse-labeling studies (16). However, acetate was not an intermediate in either carbon assimilation or methane formation. This is in agreement with our findings that none of the *Methanobacterium* species tested in our study produced extracellular acetate.

Pantskhava (14) reported that a thermophilic culture of "*Methanobacillus kuzneceovii*" produced significant amounts of acetate from methanol, CO_2, formic acid, and formaldehyde. However, Zhilina et al. (27) later showed that

TABLE 2. Relationship between methane production, acetate production, and yield of *Methanosarcina* spp[a]

Strain	Substrate[b]	Production (per liter) of:		Yield mg of cells/mmol of CH_4	μmol of acetate produced per:	
		CH_4 (mmol)	Acetate (μmol)		mg of cells	mmol of CH_4
M. barkeri MS	TMA	27.45 (1.55)	1,245 (312)	5.56 (0.05)	8.17 (1.59)	45.36 (8.43)
	MeOH	8.55 (1.66)	330 (11)	5.60 (0.15)	6.99 (0.96)	39.16 (6.40)
M. barkeri 227	TMA	29.34 (1.10)	1,532 (42)	6.54 (1.25)	8.13 (1.47)	52.20 (0.55)
	MeOH	9.33 (1.20)	336 (48)	4.61 (1.87)	8.26 (1.72)	36.7 (9.88)
M. mazei S-6	TMA	30.77 (0.71)	271 (25)	6.50 (0.15)	1.35 (0.13)	8.80 (0.91)
	MeOH	32.76 (2.35)	254 (168)	6.58 (0.41)	1.22 (0.90)	7.94 (5.66)

[a] Values in parentheses are standard deviations. $n = 3$.
[b] TMA, Trimethylamine; MeOH, methanol.

"*M. kuzneceovii*" was a mixed culture of *M. thermoautotrophicum*, autotrophic acetogenic bacteria, and heterotrophs. The possibility that acetogenic contaminants such as *Acetobacterium* or *Clostridium* species were responsible for acetogenesis in our cultures was ruled out because of the following findings. (i) Acetate was not produced when H_2-CO_2-grown cultures were inhibited with 2-bromoethanesulfonic acid. (ii) Acetate and methane were always present at the same relative concentrations when the primary substrate was exhausted. (iii) Acetate was never formed in medium containing 2 g each of Trypticase-peptone and yeast extract but having no primary substrate. (iv) The culture purity was repeatedly examined and confirmed as axenic by microscopic examination and also by culturing in complex medium.

M. barkeri is the most physiologically versatile methanogen described: it may grow chemoautotrophically on H_2-CO_2; chemoheterotrophically on methanol, trimethylamine, or acetate; or mixotrophically on CO_2-methanol-trimethylamine. Activated acetic acid or acetyl coenzyme A is the major metabolic intermediate for CO_2 assimilation in methanogens (8, 21). One possible explanation for the occurrence of acetate in our study may be the formation of such assimilatory intermediates. Amino acids and other compounds (22, 26, 28), perhaps also including acetate, may leak through the cell membrane. The differences between the two *Methanosarcina* species and the lack of significant acetate excretion in the two *Methanobacterium* species may be due to differences in metabolism, cytoplasmic acetate concentrations, or permeability of or transport across the membrane.

Our findings add to the range of compounds either excreted or leaked by *Methanosarcina* spp. into the culture medium. Because acetate is so central in assimilatory reactions, its production extends the scope of commensalistic and mutualistic relationships among the anaerobic bacteria present in the mixed methanogenic enrichments. The growth of many heterotrophic anaerobes, metabolizing a variety of substrates as energy sources, may be stimulated by acetate even at low concentrations. In marine sediments in which sulfate concentration is high, methylated amines (and perhaps methanol) are probably the most important substrates for methanogenesis (9). Under these conditions, acetate-decarboxylating, sulfate-reducing bacteria might utilize any extracellular acetate present both for energy and for assimilatory purposes. Whether methanogens are affected by an active uptake of acetate from their local surroundings requires further investigations.

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