APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1989, p. 977–983
0099-2240/89/040977-07$02.00/0

Vol. 55, No. 4

Effects of Organic Acid Anions on the Growth and Metabolism of *Syntrophomonas wolfei* in Pure Culture and in Defined Consortia

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Received 22 April 1988/Accepted 25 January 1989

The effects of organic acid anions on the growth of *Syntrophomonas wolfei* was determined by varying the initial concentration of the acid anion in the medium. The addition of 15 mM acetate decreased the growth rate of a butyrate-catabolizing coculture containing *Methanospirillum hungatei* from 0.0085 to 0.0029 per hour. Higher initial acetate concentrations decreased the butyrate degradation rate and the yield of cells of *S. wolfei* per butyrate degraded. Inhibition was not due to the counter ion or the effect of acetate on the methanogen. Initial acetate concentrations above 25 mM inhibited crotonate-using pure cultures and cocultures of *S. wolfei*. Benzoate and lactate inhibited the growth of *S. wolfei* on crotonate in pure culture and coculture. Lactate was an effective inhibitor of *S. wolfei* cultures at concentrations greater than 10 mM. High concentrations of acetate and lactate altered the electron flow in crotonate-catabolizing cocultures, resulting in the formation of less methane and more butyrate and caproate. The inclusion of the acetate-using methanogen, *Methanosarcina barkeri*, in a methanogenic butyrate-catabolizing coculture increased both the yield of *S. wolfei* cells per butyrate degraded and the efficacy of butyrate degradation. Butyrate degradation by acetate-inhibited cocultures occurred only after the addition of *Methanosarcina barkeri*. These results showed that the metabolism of *S. wolfei* was inhibited by high levels of organic acid anions. The activity of acetate-using methanogens is important for the syntrophic degradation of fatty acids when high levels of acetate are present.

Our understanding of the complete digestion of complex organic matter in methanogenic environments has been greatly facilitated by the ability to study the organisms involved in the process in defined consortia containing known species (26). The discovery of interspecies H_2-transfer-dependent catabolism (10) and the numerous reports of obligate syntrophic interactions (2, 8, 12, 14, 16, 19, 29, 30, 32, 38, 40) show that the degradation of reduced organic compounds such as alcohols, fatty acids, and some aromatic compounds in methanogenic ecosystems requires the coordinated activity of two major metabolic groups of bacteria (26). *Syntrophomonas wolfei* is an interspecies H_2-transfer-dependent, proton-reducing bacterium that catabolizes short-chain fatty acids only in association with H_2-using bacteria (29, 30, 45). It grows very slowly in coculture with *Methanospirillum hungatei* with butyrate as the energy source, since the degradation of butyrate by coculture releases a very small amount of free energy (42):

$$2\ \text{butyrate}^- + HCO_3^- \rightarrow 4\ \text{acetate}^- + CH_4,\ \Delta G_0 = -39.4\ \text{kJ} \quad (1)$$

Assuming that a free energy change of about 45 kJ mol^{-1} is required to synthesize 1 mol of ATP (42) and considering the fact that the free energy released during butyrate degradation must support the growth of two organisms, little energy is available for the growth of *S. wolfei*. Also, it has been proposed that energy is required to produce H_2 from electrons generated in the oxidation of butyryl-coenzyme A to crotonyl-coenzyme A (43), suggesting that even less of the energy generated in butyrate metabolism is available to support the growth of *S. wolfei*.

Volatile fatty acids (VFAs) were recognized in the 1930s as key intermediates in anaerobic digestion (11). Under normal conditions, acetate rarely accumulates to concentrations greater than 1 to 10 mM during active digestion (22, 36, 39), although its degradation accounts for about 70% of the methane formed (21, 39). Other VFAs are usually found in lower concentrations. However, high levels of VFAs do accumulate when anaerobic digestors are stressed by high organic loading rates or short retention times, and their accumulation often leads to digestor failure (33). The direct addition of either the ammonium or potassium salts of acetate, propionate, or butyrate to anaerobic digestor sludge decreased gas production, indicating the toxicity of these compounds (36). McCarty and McKinney (27) observed that various acetate salts inhibited acetate consumption by digestor sludge and concluded that the cation was responsible for the toxicity. These experiments were performed with acetate-fed digestors, and the results may not be indicative of complete fermentation when fermentative bacteria, H_2-producing proton-reducing bacteria, and methanogenic bacteria participate. Also, since most of the studies done on the toxicity of VFAs have used digestor sludge, it is not clear whether all or only one of the major metabolic groups are affected.

Until recently, fatty acid-degrading syntrophic bacteria could be grown only in coculture with an H_2-using bacterium, so it was not possible to determine whether the syntrophic bacterium or the methanogen was affected by high levels of organic acid anions. However, we have recently isolated *S. wolfei* in pure culture with crotonate as the energy source (6). Thus, it is now possible to compare the growth and metabolism of *S. wolfei* in pure culture and in defined mixed cultures to determine whether high concentrations of organic acid anions directly inhibit *S. wolfei* and whether acetate use in addition to H_2 use by methanogens is important under these conditions.

MATERIALS AND METHODS

Organisms and growth conditions. The basal medium of McInerney et al. (30) was modified by the addition of $NaSeO_2$ (50 μg/liter) and $NiCl_2 \cdot 6\ H_2O$ (30 μg/liter). *S.*

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wolfei DSM 2245B was grown in coculture with either *M. hungatei* JF1 or *Desulfovibrio* strain G11 in modified basal medium with 20 mM sodium butyrate. *Methanosarcina barkeri* was grown in pure culture in the modified basal medium with 0.2% sodium acetate and was directly added to a butyrate-catabolizing, *S. wolfei-M. hungatei* coculture. This triculture completely mineralized butyrate to CH_4 and CO_2. The pure culture of *S. wolfei* was grown in the modified basal medium with equimolar amounts of sodium crotonate replacing sodium butyrate (6).

Methods for the preparation of anaerobically prepared media were essentially those of Bryant (9). Media were dispensed into aluminum-seal culture tubes (18 by 150 mm), 100- or 125-ml serum bottles, and 1- or 2-liter bottles. The bottles were fitted with black rubber stoppers into which a sealed tube was inserted (3) to facilitate sampling and making additions to the medium. Additions, inoculations, and sampling of the media were done by using syringe and needles (3). Purity was checked by microscopic examination, inoculation into thioglycolate medium (which does not support the growth of these bacteria), and inoculation into basal medium without butyrate or crotonate. Growth was routinely measured as change in A_{600} on a Bausch & Lomb Spectronic 21 spectrophotometer (30). Generation times were estimated from the linear portion of a semilogarithmic plot of absorbance versus time and are defined as the time required to double the absorbance of the culture. Direct counting of bacteria was performed using a Petroff-Hauser counting chamber (34).

The effects of different initial concentrations of organic acid anions on the growth and metabolism of the cultures described above were studied by adding different amounts of the sodium or potassium salts of the acids to the modified basal medium before autoclaving. Appropriate amounts of NaCl or KCl were added so that the ionic strength of each medium was equal to that of the medium with the highest amount of the organic acid salt.

Analytical methods. Acetate, butyrate, crotonate, and caproate were determined as free fatty acids by gas chromatography by using packed (37) or capillary (20) columns. Methane was measured by gas chromatography as previously described (4).

Whole-cell protein was determined by collecting the cells in 3-ml samples by centrifugation (10,000 × *g*, 4°C, 10 min). The cells were washed twice in 15 ml of 50 mM phosphate buffer (pH 7.4) by centrifuging and resuspending the pellet to remove excess sulfide. The final cell pellet was suspended in 0.5 ml of 1 N NaOH and digested by boiling the suspension for 30 min. Protein was then determined by the method of Lowry et al. (25).

Lipid phosphate was determined by the method of White et al. (44) on cells harvested by centrifugation (10,000 × *g*, 4°C, 10 min). Lipids were extracted with chloroform-methanol-water (1:1:0.9). The liquid residue after evaporation of chloroform was ashed by gentle heating in the presence of $Mg(NO_3)_2$. The material was then boiled in 1N HCl for 15 min and neutralized, and the amount of phosphate present was determined.

RESULTS

Effect of acetate on butyrate-degrading cocultures. To determine the effect of acetate on growth, the *S. wolfei-M. hungatei* coculture was grown in butyrate basal medium with different initial concentrations of sodium acetate from 15 to 65 mM (data not shown). In the unsupplemented medium, the coculture grew with a specific growth rate of 0.0085 per h. The addition of 15 mM sodium acetate to the medium decreased the growth rate by 66%. At higher initial acetate concentrations, growth was too erratic to determine growth rates. The total amount of growth attained by the coculture decreased by at least 50% when the coculture was grown in medium with an initial acetate concentration of 15 mM or higher. When very high initial acetate concentrations were used, little or no growth occurred although some butyrate was degraded (Table 1). Similar results were obtained when the amount of biomass was determined by lipid phosphorous content (data not shown).

TABLE 1. Effects of high acetate concentrations on the growth of *S. wolfei-M. hungatei* coculture[a]

Acetate added (mM)	Percent butyrate degraded (mean ± SD)	Protein concn (g/liter) (mean ± SD)	Yield (g of protein/mol of butyrate) (mean ± SD)
None	65.3 ± 3.68	23.5 ± 4.03	0.68 ± 0.064
88	47.1 ± 0	6.8 ± 0.50	0.28 ± 0.014[b]
135	25.1 ± 12.5	2.8 ± 0.52	0.27 ± 0.17[b]

[a] Two individual cultures containing 500 ml of basal medium with 50 mM sodium butyrate were inoculated with 10 ml of the coculture. The cultures were analyzed after 504 h of incubation.

[b] Significantly different from control at $P \leq 0.05$ by using a two-tailed *t* test.

A sharp decrease in the rate of butyrate use was observed with the addition of 10 to 15 mM acetate (Fig. 1). The rate of butyrate degradation continued to decrease in cultures with initial acetate concentrations of 20 to 55 mM. When the initial acetate concentration was 55 mM, the rate of butyrate use was only 35% of that when the control without acetate was added. Similar results were observed when the coculture as grown in medium amended with potassium acetate (data not shown). The addition of 40 mM potassium acetate decreased the growth rate and total amount of growth of the coculture by 56 and 24%, respectively. This indicated that acetate and not the counter ion inhibited the growth and butyrate degradation of the coculture. Axenic cultures of *M. hungatei* grown in the same medium with H_2-CO_2 as the energy source were stimulated by the addition of up to 150 mM acetate to the medium (data not shown), indicating that acetate inhibited the growth and metabolism of *S. wolfei* and not that of *M. hungatei*.

The effect of acetate on crotonate-catabolizing cultures. To confirm that acetate inhibited *S. wolfei*, the growth and metabolism of *S. wolfei* grown in pure culture on crotonate with varying initial acetate concentrations was studied. The growth rate of pure cultures of *S. wolfei* decreased 58% with the addition of 25 mM acetate to the medium (Table 2). The total amount of growth progressively decreased as the initial acetate concentration varied from 0 to 100 mM. When the initial acetate concentrations were above 50 mM, very long lag times were observed, ranging from 45 to 135 days. However, these higher initial acetate concentrations decreased the growth rate only slightly compared with an initial acetate concentration of 25 mM. The growth of an *S. wolfei-M. hungatei* coculture on crotonate was also inhibited by the addition of high initial acetate concentrations (Table 3). Increasing the initial amount of acetate decreased both the growth rate and the total amount of growth and increased the lag time of the *S. wolfei-M. hungatei* coculture to a greater extent than it did with the pure culture of *S. wolfei*.

The addition of acetate also changed the fermentation pattern of the *S. wolfei-M. hungatei* coculture. In pure

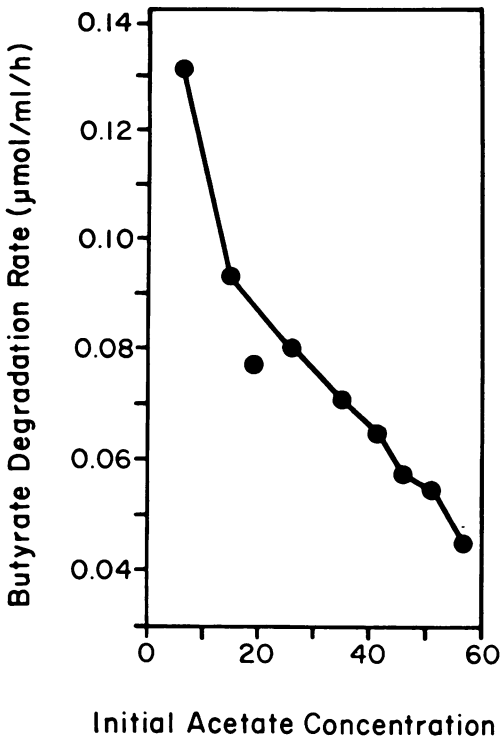

FIG. 1. Effect of varying the initial acetate concentration on the rate of butyrate degradation by an *S. wolfei-M. hungatei* coculture. Butyrate degradation rate was determined as the maximum slope obtained from a plot of butyrate concentration versus incubation time. Complete butyrate degradation required 11 to 20 days, depending on the initial acetate concentration. The basal medium was amended with anoxic stock solutions of sodium acetate (pH 7) or sodium chloride. The initial acetate concentration was determined immediately after inoculation. Each point represents an independent observation.

culture, varying the initial acetate concentration did not affect the amount of crotonate used or the products formed from crotonate by *S. wolfei* (Fig. 2). However, varying the initial acetate concentration decreased the amount of crotonate degraded by the coculture and shifted the fermentation pattern of the coculture. When the initial acetate concentration was greater than 50 mM, the coculture produced less methane and more nongaseous products (butyrate and caproate). At 100 mM acetate, butyrate and caproate accounted for 45% of the electron equivalents generated from crotonate.

The effects of other organic acid anions on crotonate-catabolizing cultures of *S. wolfei*. The addition of benzoate or lactate to the medium inhibited the growth of the pure cultures and cocultures of *S. wolfei* (Tables 2 and 3). The growth rates of the pure cultures and cocultures of *S. wolfei* decreased by 50 and 65%, respectively, when the initial benzoate concentration was 50 mM. When the initial benzoate concentration was 100 mM, the growth rates of the pure cultures and cocultures decreased 80 and 95%, respectively. The addition of 10 to 20 mM lactate was very inhibitory to the growth of both the pure cultures and cocultures. The pure culture of *S. wolfei* was more sensitive to lactate inhibition than the coculture. No growth of the pure culture was observed in medium with more than 50 mM lactate, while some growth of the coculture was observed in medium with up to 100 mM lactate. Similar results were

TABLE 2. Effects of acetate, benzoate, and lactate on the growth of *S. wolfei* in pure culture in crotonate medium[a]

Addition	Concn (mM)	Lag time (h)[b]	Growth rate (h^{-1})	Growth (A_{600})[c]
Acetate	0	0	0.015	0.35
	25	0	0.0087	0.31
	50	45	0.0081	0.29
	75	45	0.0073	0.23
	100	135	0.0073	0.18
Benzoate	0	0	0.015	0.35
	25	20	0.0077	0.29
	50	20	0.0069	0.28
	75	40	0.0050	0.23
	100	40	0.0029	0.17
Lactate	0	37	0.020	0.55
	10	37	0.027	0.50
	20	85	0.010	0.35
	30	85	0.007	0.19
	40	—[d]	—	0.10
	50	—	—	0.07

[a] Sodium salts of acetate and benzoate and lithium lactate were added at the indicated concentration.
[b] Mean of three independent cultures.
[c] Maximal change in absorbance.
[d] —, Absorbance changes too small to determine accurately.

obtained when the lithium salt of lactate was used. As was the case for acetate, varying the initial concentrations of both benzoate and lactate greatly increased the lag times of the pure culture and the coculture of *S. wolfei* (Tables 2 and 3).

Although high initial lactate concentrations (75 to 100 mM) inhibited the growth of the *S. wolfei-M. hungatei* coculture, the cocultures were metabolically active as determined by methane and butyrate production. High lactate concentrations altered electron flow in the same manner as high acetate concentrations. In cocultures with 100 mM lactate, methane accounted for only 66% of the reducing equivalents

TABLE 3. Effects of acetate, benzoate, and lactate on the growth of *S. wolfei* in coculture with *M. hungatei* in crotonate medium[a]

Addition	Concn (mM)	Lag time (h)[b]	Growth rate (h^{-1})	Growth (A_{600})[c]
Acetate	0	0	0.012	0.38
	20	20	0.009	0.34
	50	40	0.005	0.22
	75	110	0.004	0.17
	100	240	0.003	0.09
Benzoate	0	0	0.012	0.38
	20	0	0.001	0.38
	50	90	0.004	0.26
	75	110	0.002	0.12
	100	240	0.001	0.04
Lactate	0	0	0.012	0.38
	20	20	0.009	0.31
	50	200	0.005	0.24
	75	250	0.002	0.06
	100	350	0.0005	0.04

[a] Sodium salts of acetate and benzoate and lithium lactate were added at the indicated concentration.
[b] Mean of three independent cultures.
[c] Maximal change in absorbance.

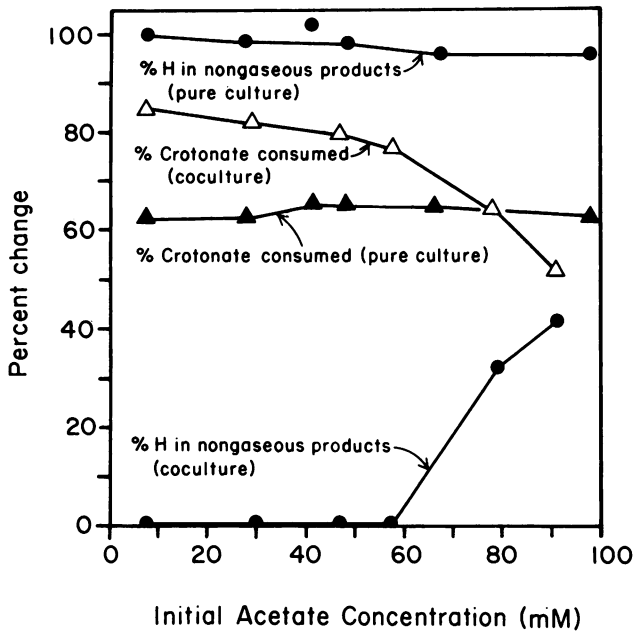

FIG. 2. Effect of varying the initial acetate concentration on the growth and degradation of crotonate by *S. wolfei* grown alone or in coculture with *M. hungatei*.

generated from crotonate compared with cocultures containing lower initial lactate concentrations (50 mM or less) in which methane accounted for 93% of the reducing equivalents.

Effect of an acetate-using methanogen on the growth and metabolism of *S. wolfei*. *Methanosarcina barkeri*, an aceto-clastic methanogenic bacterium, was added to the coculture of *M. hungatei* and *S. wolfei*. This triculture completely metabolized butyrate to CO_2 and CH_4. Compared with the coculture containing *S. wolfei* with *Desulfovibrio* strain G11, the triculture degraded more butyrate before growth ceased (Table 4). The yield of *S. wolfei* cells (per micromole of butyrate degraded) increased twofold in the triculture compared with the coculture, suggesting that the accumulation of acetate decreased the ability of *S. wolfei* to couple butyrate degradation to energy conservation.

TABLE 4. Effect of *Methanosarcina barkeri* on the growth of *S. wolfei* in coculture with *Desulfovibrio* strain G11 in butyrate medium[a]

Culture[b]	Growth rate (h^{-1})	Butyrate degraded (mM)[c]	*S. wolfei* (cells/ml)[d]	Yield (*S. wolfei* cells/mol of butyrate)
Coculture	0.005	26.0 ± 0.1	2.4×10^7 ± 13%	9.1×10^{11}
Triculture	0.004	34.4 ± 0.6	6.1×10^7 ± 10%	1.8×10^{12}

[a] One milliliter of the respective culture was inoculated into 10 ml of basal medium with 40 mM sodium butyrate. Ninety-two percent of the butyrate carbon that was degraded was recovered as acetate in the coculture, while only 67% was recovered as acetate in the triculture.
[b] The coculture contained *S. wolfei* with *Desulfovibrio* strain G11. The triculture contained *S. wolfei*, *M. hungatei*, and *Methanosarcina barkeri*.
[c] Mean of two independent cultures ± the standard deviation.
[d] Mean of two independent cultures ± the coefficient of variation.

In a separate experiment, the triculture was compared with a coculture containing *M. hungatei*. Both of these cultures metabolized 90% of the butyrate present within 560 h. A stoichiometric accumulation of acetate was observed (Fig. 3). After 340 h, the cell yields of *S. wolfei* were slightly higher in the triculture (3.5×10^6 cells per μmol of butyrate) compared with the coculture (2.5×10^6 cells per μmol of butyrate). The cell yields of *M. hungatei* were similar in both cultures, 2.2×10^6 and 2.3×10^6 cells per μmol of butyrate, respectively. *Methanosarcina barkeri* was not detected microscopically until 360 h after inoculation. Small clumps of *Methanosarcina barkeri* (about 2.5×10^5 clumps per ml) and acetate degradation were observed at this time. Each culture was amended with a neutralized solution of butyric acid at 530 h to bring the concentration of butyrate in each culture to 25 mM. Butyrate use began in the triculture within 100 h. The concentration of acetate in the triculture did not change during this period, indicating that the additional acetate produced from butyrate was metabolized. The amount of acetate present at 1,300 h accounted for 34% of the total amount of butyrate carbon metabolized since inoculation. The triculture received a third addition of butyrate, which it metabolized by 2,200 h. No butyrate degradation was observed in the *S. wolfei-M. hungatei* coculture during this period. The addition of *Methanosarcina barkeri* to the coculture resulted in butyrate degradation within 100 to 200

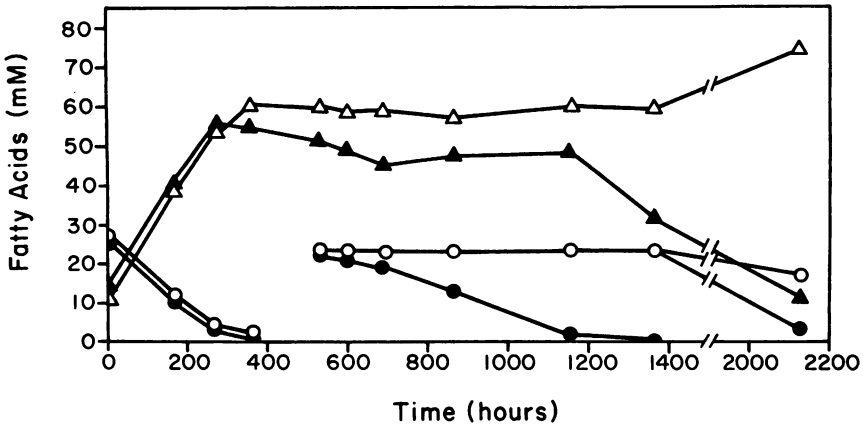

FIG. 3. Effect of the addition of *Methanosarcina barkeri* on butyrate metabolism by the *S. wolfei-M. hungatei* coculture. Initial butyrate concentration was 30 mM. At 530 h, a reduced sodium butyrate solution was added to each culture to bring the butyrate concentration to 25 mM. The triculture received another 25 mM addition of butyrate at 1,400 h. Symbols: ▲ and ●, triculture; △ and ○, coculture; △ and ▲, acetate concentration; and ○ and ●, butyrate concentration.

h after the addition (data not shown). These data showed that the addition of an acetate-using bacterium allowed butyrate to be degraded by *S. wolfei* when the acetate concentration was at a level that inhibited butyrate degradation in cultures without the acetate user. The fact that butyrate degradation occurred at acetate concentrations that inhibited cocultures which could not actively metabolize acetate suggests that the turnover of acetate as well as its concentration relative to butyrate influences the activity of *S. wolfei*.

To exclude the possibility that *Methanosarcina barkeri* may have produced a growth factor which stimulated the growth of *S. wolfei*, culture fluid from the triculture was added to the butyrate basal medium used to grow the *S. wolfei-M. hungatei* coculture. The addition of up to 40% cell-free triculture fluid to the medium prior to autoclaving failed to stimulate the growth of the coculture.

DISCUSSION

The methanogenic digestion of complex organic matter is a three-stage process in which the proton-reducing acetogenic bacteria play an important role (28). However, little is known about how these bacteria respond to high organic acid anion concentrations, which often occur when digestors are stressed by high organic loading rates. In this study, we showed that high concentrations of organic acid anions at neutral pH inhibited the growth and metabolism of *S. wolfei* in pure culture and in association with H_2-using bacteria. This disagrees with McCarty and McKinney (27), who concluded that it was the high concentration of the counter ion which was responsible for the inhibition. Since our experiments were conducted at constant ionic strength and pH, the inhibitory effects can be attributed to the dissociated form of the acid and not to the counter ion or changing ionic conditions. The observation that acetate inhibits benzoate degradation by a coculture (13) and propionate degradation both in anaerobic digestor sludge (23) and in a coculture containing *Syntrophobacter wolinii* (8) suggests that acetate is inhibitory to other syntrophic bacteria. These results confirm the conclusion of Buswell (11) that intermediary metabolism in methanogenic digestion is sensitive to acetate concentrations greater than 30 mM. These data also suggest that pH control alone may not be sufficient in counteracting the inhibitory effects of high concentrations of organic anions. Instead, organic loading rates should be adjusted to prevent the accumulation of high concentrations of organic acids.

The presence of an acetate-using methanogen in a triculture which also contained *S. wolfei* and *M. hungatei* allowed butyrate degradation to occur at acetate concentrations which inhibited a coculture of *S. wolfei* and *M. wolfei*. This indicates that acetate turnover is important for the syntrophic degradation of butyrate by *S. wolfei* when high levels of acetate are present. However, since the concentration of acetate in the triculture was about 50 mM compared with about 60 mM in the inhibited coculture, it may be argued that *Methanosarcina barkeri* lowered the acetate concentration enough to relieve end-product inhibition and shift the equilibrium of the reaction so that butyrate degradation was thermodynamically possible under these conditions. In either case, the continued use of acetate is still required to prevent further accumulation of acetate. The reason why butyrate degradation was inhibited at concentrations of 60 mM in some cocultures (Fig. 3), while some butyrate degradation occurred in other cocultures with much higher initial acetate concentrations (Table 1), may be due to the differences in the physiological state of the cultures. The cocultures used in the experiment shown in Fig. 3 were in stationary phase while those used for experiments described in Table 1 were in exponential phase.

Ahring and Westermann (1) found that the rate of butyrate use was faster in a triculture containing a thermophilic butyrate-degrading syntrophic bacterium, an H_2-using methanogen, and an acetate-using methanogen than in the coculture which lacked the acetate-using methanogen. We found that the addition of *Methanosarcina barkeri* increased the growth yield of *S. wolfei* with butyrate, suggesting that *S. wolfei* was able to derive more energy from butyrate metabolism when an acetate-using organism was present. This was not due to the production of a growth factor by *Methanosarcina barkeri*, since the addition of fluids of tricultures with *Methanosarcina barkeri* did not stimulate the growth of *S. wolfei* in cocultures with *M. hungatei*. However, definite conclusions in this regard are difficult to draw, since the differences in the growth yields were not always significant because of the ineffective coupling of butyrate catabolism to acetate consumption. It would be interesting if higher growth yields of *S. wolfei* are observed when a *Methanothrix* species is used, since this methanogen has a greater affinity for acetate than does *Methanosarcina barkeri*.

Accumulation of acetic and butyric acids has been recognized as a primary factor contributing to the onset of solventogenesis by solvent-producing clostridia (15, 17). Higher concentrations of these acids are required to shift the fermentation toward solventogenesis at neutral pH compared with the concentration needed at an acidic pH. The enhanced effect associated with low pH is apparently due to increased membrane permeability by the undissociated form of these acids (18). The presence of high organic acid concentrations at low pH could result in energetic stress created by rapid equilibration of a proton gradient (5, 15). It has been suggested that *S. wolfei* requires a proton motive force to produce H_2 from electrons generated from the oxidation of butyryl-coenzyme A to crotonyl-coenzyme A (43). The high organic acid concentrations could inhibit this process by dissipating the proton motive force. However, Kell et al. (24) demonstrated that clostridial membranes are relatively impermeable to acetate at neutral pH. This, plus the fact that relatively moderate concentrations of acetate (20 to 60 mM) are inhibitory to *S. wolfei*, suggests that the mode of inhibition may not be due simply to the protonophoric activity of acetate. Also, butyrate degradation and enhanced growth of *S. wolfei* occurred at acetate concentrations of 50 to 60 mM in tricultures containing *Methanosarcina barkeri* but not in cocultures without the acetate user. If the mode of inhibition was due solely to the protonophoric activity of acetate, then both cultures should have responded similarly, since the acetate concentrations in each culture were similar. The protonophore, carbonyl *p*-chlorophenylhydrazone, at concentrations up to 100 μM only slightly inhibited the growth and did not affect the fermentation pattern of *S. wolfei* grown with crotonate (P. S. Beaty and M. J. McInerney, unpublished data). This suggests that *S. wolfei* is relatively insensitive to the action of protonophores. The inhibitory effect of acetate may be explained from a thermodynamic viewpoint, since high concentrations of an end product of metabolism would inhibit further end product formation. However, other organic acids which are not end products of butyrate or crotonate metabolism also inhibit the growth and metabolism of *S. wolfei*. This, plus the fact that butyrate degradation occurred in tricultures but not

in cocultures, even though both cultures had similar acetate concentrations, is inconsistent with acetate inhibition being solely the result of end product inhibition.

The accumulation of high intracellular concentrations of acetate and butyrate has been reported in *Clostridium acetobutylicum* (41). This suggests that it may be possible to generate an electrochemical potential via the electrogenic efflux of these end products, as found with lactate excretion in streptococci (31). The energetic efflux of lactate has been shown to provide up to 30% of the energy needed for growth during the homolactic fermentation of glucose by *Streptococcus lactis* (35). The formation of an acetate gradient during butyrate catabolism by *S. wolfei* is theoretically possible (7). The very low free-energy changes associated with anaerobic butyrate degradation (30, 43) make this an attractive hypothesis. The enhancement of the growth yield in the presence of the acetoclastic methanogen suggests that an increase in energy efficiency occurs when acetate turnover is maintained. At this time, no direct experimental evidence for this system exists.

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