APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1988, p. 2393–2397
0099-2240/88/102393-05$02.00/0

Vol. 54, No. 10

Product Inhibition of Butyrate Metabolism by Acetate and Hydrogen in a Thermophilic Coculture

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Received 4 April 1988/Accepted 8 July 1988

Studies on product inhibition of a thermophilic butyrate-degrading bacterium in syntrophic association with *Methanobacterium thermoautotrophicum* showed that a gas phase containing more than 2×10^{-2} atm (2.03 kPa) of hydrogen prevented growth and butyrate consumption, while a lower hydrogen partial pressure of 1×10^{-3} to 2×10^{-2} atm (0.1 to 2.03 kPa) gradually inhibited the butyrate consumption of the coculture. No inhibition of butyrate consumption was found on the addition of 0.75×10^{-3} atm (76 Pa) of hydrogen to the gas phase. A slight inhibition of butyrate consumption by the coculture occurred at an acetate concentration of 16.4 mM. Inhibition gradually increased with increasing acetate concentration up to 81.4 mM, when complete inhibition of butyrate consumption occurred. When the culture contained an acetate-utilizing methanogen in addition to *M. thermoautotrophicum*, the inhibition of the triculture by acetate was gradually reversed as the acetate concentration was lowered by the aceticlastic methanogen. The results show that optimal growth conditions for the thermophilic butyrate-degrading bacterium depend on both hydrogen and acetate removal.

Anaerobic degradation of organic matter in the absence of light, nitrate, oxygen, and sulfate involves the complex metabolic interactions of at least three entirely different groups of bacteria: the fermentative bacteria, the obligate proton-reducing acetogenic bacteria, and the methanogenic bacteria (8, 12, 16, 18). Acetate and hydrogen are the principal products of the first two metabolic groups of bacteria and are metabolized by the hydrogen-oxidizing and aceticlastic methanogens, respectively (22, 25, 26). The concentration of hydrogen and, hence, the activity of the hydrogen-oxidizing methanogenic bacteria may regulate the fermentative pathways of the fermentative bacteria. If the concentration of hydrogen is low, the fermentation products shift from a more reduced to a more oxidized form, as reported from numerous experiments with pure and mixed cultures fermenting carbohydrates (for a review, see reference 17). The obligate proton-reducing acetogenic bacteria do not exhibit such a shift in oxidation state of the products, and growth and metabolism only occur if the hydrogen partial pressure is sufficiently low to make these reactions thermodynamically feasible (8, 19, 20, 23). Based on thermodynamic calculations, it can be estimated that the hydrogen partial pressure must be maintained at a level of 10^{-5} to 10^{-6} atm (0.1 to 1.0 Pa) to allow oxidation of propionate and butyrate (18). The concentration of acetate may also affect the oxidation of propionate and butyrate, although much higher concentrations are needed compared with the concentration of hydrogen that is needed. Only a few studies have been conducted to determine the actual effects of product inhibition on the mesophilic degradation of butyrate or propionate. The aim of the present study was to examine the influence of hydrogen and acetate on the butyrate consumption of a thermophilic butyrate-degrading bacterium in syntrophic association with methanogens.

(Portions of this study have been presented elsewhere [B. K. Ahring and P. Westermann, Abstr. Proc. 4th European Congr. Biotechnol., 1987, vol. 3, p. 510].)

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MATERIALS AND METHODS

Source of organisms. The isolation procedure for the thermophilic butyrate-degrading bacterium has been reported previously (2, 3). The butyrate-degrading bacterium was isolated in coculture with *Methanobacterium thermoautotrophicum* or in triculture with both *M. thermoautotrophicum* and the TAM organism, a thermophilic acetate-utilizing methanogenic bacterium. The initial inoculum was obtained from a thermophilic (60°C) bench-scale digestor operating on sewage sludge.

Media and conditions for cultivation. Strict anaerobic techniques developed by Hungate (11), Bryant (7), and Balch and Wolfe (4) were used throughout this investigation. The medium for cultivation of the coculture and the triculture was basically the sulfate-free medium used for enrichment and isolation of the thermophilic butyrate-degrading bacterium used previously (2). The butyrate concentration was 10 mM (added as sodium butyrate), and the medium was supplemented with 0.1% yeast extract. Experiments were performed either in serum vials (50 ml, containing 25 ml of medium) closed with butyl rubber stoppers and aluminum crimps or in serum bottles (500 ml, containing 250 ml of medium) closed with black butyl rubber stoppers. Unless stated otherwise, the gas phase was 80% N_2–20% CO_2 pressurized to 1 atm (101.3 kPa) of overpressure in the vials or constantly supplied at a gassing rate of 200 ml/min in the serum bottles. The vials were incubated in static cultures, while the serum bottles were constantly stirred (500 rpm) with a magnetic stirring bar during the incubation period. A scheme of the experimental setup of the serum bottles is shown in Fig. 1. All vessels were incubated at 60°C at least in duplicate, and the data were averaged.

Growth of the cocultures. Growth of the cocultures was defined in terms of the specific butyrate consumption rate (μ_{but}), which was calculated as the slope of the linear part of the half-logarithmic graph of the butyrate concentration versus time.

Effect of addition of hydrogen and acetate. The effects of the addition of hydrogen and acetate on the butyrate consumption of exponentially growing cocultures of the thermo-

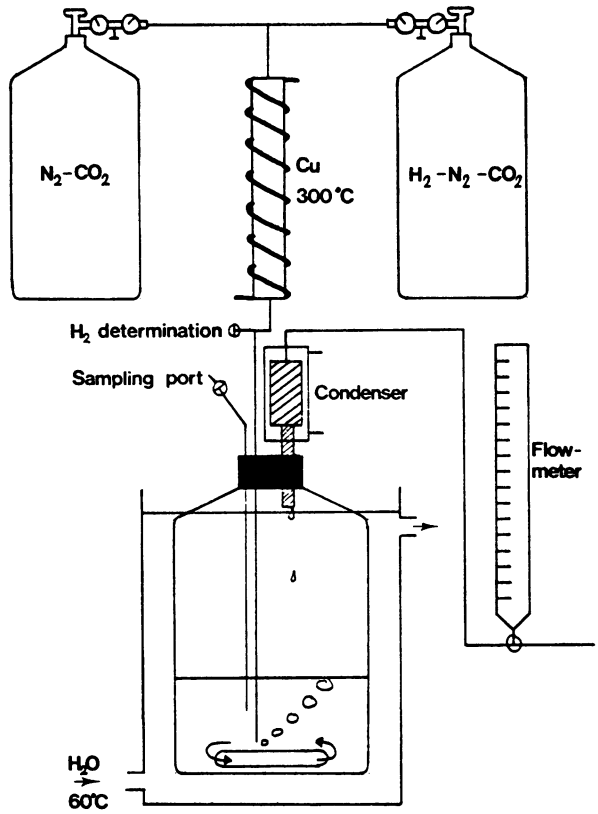

FIG. 1. Experimental setup for determination of the butyrate consumption rates under various gas phases.

philic butyrate-degrading bacterium in coculture with *M. thermoautotrophicum* were studied in serum bottles and serum vials, respectively. All experiments were initiated when the cocultures were at the same stage of growth, and a concentration of approximately 4.8 and 6.8 mM butyrate was left in the serum bottles and serum vials, respectively.

For the experiments with hydrogen, the appropriate partial pressure of hydrogen (0.075×10^{-2}, 0.1×10^{-2}, 0.5×10^{-2}, 1.0×10^{-2}, 2.0×10^{-2}, 3.0×10^{-2}, 5.0×10^{-2} atm [1 atm = 101.3 kPa]) was added to the in-flowing gas by mixing 80% N_2–20% CO_2 with 5% H_2–75% N_2–20% CO_2. The exact concentration was checked by gas chromatography. The butyrate consumption was followed by obtaining measurements at 1-h intervals. The pH remained between 7.1 and 7.3.

For the experiments with acetate, the appropriate acetate concentrations (10, 25, 50, 75, and 100 mM) were added as sodium acetate to exponentially growing cultures. The exact concentration in the cocultures after the addition of acetate was checked by gas chromatography before and after the addition. Because of the metabolism of butyrate, a concentration of approximately 6.4 mM acetate was present in the coculture when the experiments were initiated, and acetate at this concentration must be added to give the final concentration of acetate in the culture. Butyrate consumption was followed by obtaining two daily measurements.

The reversibility of the inhibition of butyrate consumption with acetate was studied in serum vials with exponentially growing cultures of the butyrate-degrading bacterium together with *M. thermoautotrophicum* and TAM. A slug of neutralized acetate, resulting in a total concentration of 82.4 mM acetate in the tricultures, was added; and butyrate and acetate consumption was followed by obtaining measurements at appropriate intervals.

TABLE 1. Specific butyrate consumption rate of the thermophilic butyrate-degrading coculture under various growth conditions

Growth conditions[a]	μ_{but} (h^{-1})[b]
Static culture	0.0295 ± 0.002
Shaking (500 rpm)	0.0615 ± 0.008
Shaking (500 rpm) and gassing (200 ml/min)	0.0695 ± 0.004

[a] Experiments were performed in batch cultures in 500-ml serum bottles with 250 ml of medium under the indicated growth conditions.
[b] μ_{but}, Specific butyrate consumption rate. Values are means ± standard deviations of triplicate determinations.

Analytic procedures. Acetate and butyrate were detected by gas chromatography with a flame ionization detector as described previously (1). Hydrogen was detected by thermal conductivity as described previously (3).

Gases and chemicals. High-purity gases were used, and traces of oxygen were removed by passing the gases over an oxygen scrubber (O-trap; Supelco) or through a copper column.

RESULTS

Butyrate consumption by the coculture and the triculture. The butyrate-degrading bacterium grown in coculture with *M. thermoautotrophicum* metabolized butyrate and acetate and methane were formed, while the triculture containing the additional acetate-utilizing methanogen completely degraded butyrate to methane and carbon dioxide. Butyrate consumption occurred at a higher rate in the triculture than in the coculture, as described previously (2). The specific butyrate consumption rate was slightly higher when the cocultures were cultivated in larger volumes. However, the specific butyrate consumption rate was doubled when the cocultures were incubated with intensive shaking, and the rate was even higher when the coculture was continuously outgassed (Table 1).

Effect of hydrogen. The effect of hydrogen on the coculture is shown in Fig. 2. Hydrogen produced an immediate inhibitory effect on butyrate consumption by the coculture. A partial pressure of 3×10^{-2} atm (3.04 kPa) of hydrogen was totally inhibitory to growth and butyrate consumption, while a partial pressure of 0.075 to 2×10^{-2} gradually inhibited butyrate consumption. No inhibition was found on the addition of 0.75×10^{-3} atm (76 Pa) of hydrogen. Butyrate consumption was exponential in all experiments, even in vessels containing partially inhibited cocultures. The growth rate constants, therefore, could be determined for different cocultures that were inhibited at different percentages (Table 2). The response to hydrogen was most pronounced, while the partial pressure of hydrogen was raised slightly over the permissive range of hydrogen (from 0.1×10^{-2} to 0.5×10^{-2} atm [0.1 to 0.5 kPa]) (Table 2).

Effect of acetate. The effect of acetate on the coculture was an immediate reduction of the butyrate consumption rate by even the lowest acetate concentration tested (10 mM, corresponding to a final concentration of 16.4 mM in the vials) (Fig. 3). However, the concentrations causing an inhibitory effect were considerably higher for acetate compared with those for hydrogen. In contrast to the hydrogen experiments, inhibition of butyrate consumption increased slightly 1 day after the addition of acetate. The specific butyrate consumption rates determined for the various levels of

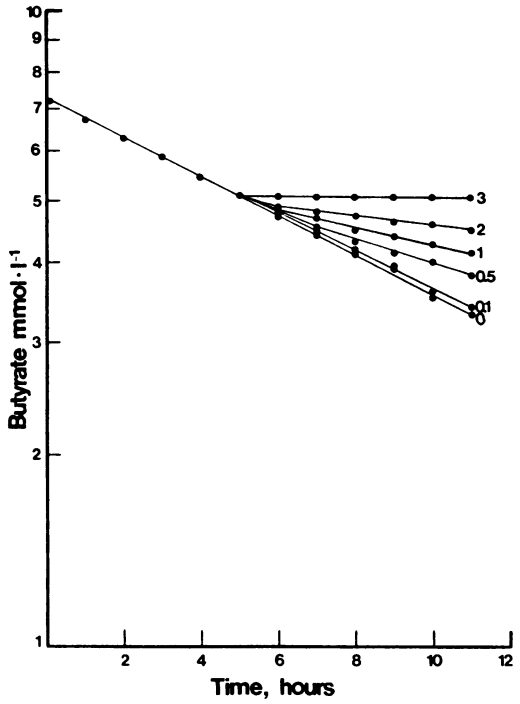

FIG. 2. Effect of hydrogen partial pressure (ranging from 0 to 3 × 10^{-2} atm) on the butyrate consumption versus time of the thermophilic butyrate-degrading coculture.

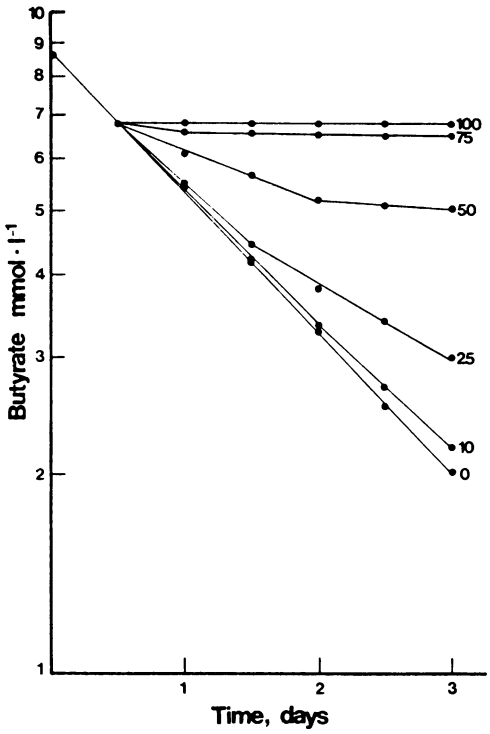

FIG. 3. Effect of acetate concentration (ranging from 0 to 100 mM) on the butyrate consumption versus time of the thermophilic butyrate-degrading coculture.

inhibition by acetate are shown in Table 3. The largest effect of acetate addition was found by increasing the added concentration from 25 to 50 mM. The addition of 75 mM acetate almost stopped all growth and butyrate consumption by the coculture, whereas 75 mM NaCl had no effect.

Acetate was utilized concurrently with its production in the triculture. When 75 mM acetate was added to the triculture, inhibition of butyrate consumption was gradually reversed when the acetate concentration was lowered by the acetate-utilizing aceticlastic methanogen (Fig. 4).

DISCUSSION

Stability of the anaerobic digestion process requires a balanced activity of the mixed populations of bacteria that are present. Operational factors that are usually associated with failure in an anaerobic digestor include a rapid increase in the rate of organic loading, rapid process temperature changes, and the presence of inhibitory compounds in the waste (1, 14, 24). The methanogenic bacteria are considered to be a very sensitive group in anaerobic digestion, and failure in the digestion process is often accredited to these organisms (1, 9, 14). Hydrogen and acetate are the principal substrates of methanogenesis in an anaerobic digestor. Results of the present study indicate that the accumulation of hydrogen and acetate can inhibit the activity of the thermophilic acetogenic bacteria that degrade butyrate in syntrophic association with methanogens.

The partial pressure of hydrogen that caused initial inhibition of butyrate consumption by the thermophilic butyrate-degrading coculture, 1 × 10^{-3} atm (0.1 kPa), is lower than

TABLE 2. Effect of hydrogen on the specific butyrate consumption rate of the thermophilic butyrate-degrading coculture[a]

Hydrogen partial pressure (10^{-2} atm)	μ_{but} (h^{-1})[b]	% Inhibition[c]
0	0.0696	
0.075	0.0697	0
0.1	0.0678	2.6
0.5	0.0472	32.2
1.0	0.0331	52.4
2.0	0.0176	74.7
3.0	0	100

[a] Experiments were performed in 500-ml serum bottles with 250 ml of exponentially growing cocultures. The partial pressure of the in-flowing gas was as indicated.

[b] μ_{but}, Specific butyrate consumption rate. Values are means of two independent experiments. The standard deviations were less than 0.005.

[c] Percent inhibition of the butyrate consumption rate compared with those of controls with no hydrogen added.

TABLE 3. Effect of acetate on the specific butyrate consumption rate of the thermophilic butyrate-degrading coculture[a]

Acetate concn (mM)	μ_{but} (h^{-1})[b]	% Inhibition[c]
0	0.0203	
10	0.0189	6.9
25	0.0135	33.5
50	0.0051	74.9
75	0.0007	96.6
100	0	100

[a] Experiments were performed in 50-ml serum vials with 25 ml of exponentially growing cocultures with an acetate concentration of approximately 6.4 mM.

[b] μ_{but}, Specific butyrate consumption rate. Values are means of three independent experiments. The standard deviations were less than 0.003.

[c] Percent inhibition of the butyrate consumption rate compared with those of controls with no acetate added.

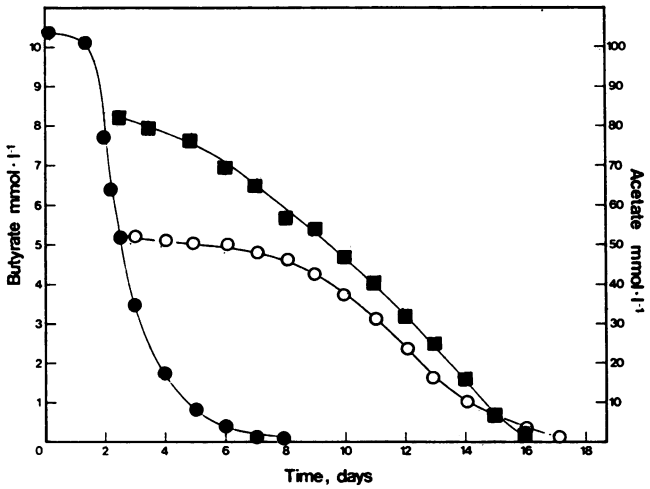

FIG. 4. Effect of acetate and acetate utilization on butyrate consumption by the thermophilic butyrate-degrading triculture. A concentration of 75 mM acetate was added to the triculture after 2.5 days of incubation. Symbols: ○, butyrate; ■, acetate; ●, butyrate in control cultures.

the corresponding partial pressure of 5×10^{-3} atm (0.5 kPa) found for inhibition of propionate oxidation in mesophilic digesting sludge (13). No measurable effect on butyrate consumption by the thermophilic coculture was observed at a hydrogen partial pressure of 0.75×10^{-3} atm (76 Pa), while the corresponding partial pressure that caused no effect on the mesophilic oxidation of propionate in sludge was 3×10^{-3} (0.3 kPa). However, the values found in our study are not directly comparable to those found in studies with mesophilic organisms, since lower concentrations of hydrogen are expected in solution under thermophilic temperatures, implying that the actual differences may be even higher. Furthermore, it may be difficult to obtain equilibration between the gas phase and the liquid phase in sludge samples because of phase transfer limitations under conditions of rapid utilization of hydrogen by the microbial populations (21).

A partial pressure of approximately 0.7×10^{-3} to 8×10^{-3} atm (71 to 182 Pa) of hydrogen was measured in the headspace of serum vials of exponentially growing cocultures. This indicates that the coculture may be slightly inhibited by hydrogen during batch cultivation and also explains why a constant outgassing of the culture increases the butyrate consumption rate by the coculture. Results of previous experiments showed that the inhibition of growth and butyrate consumption by 0.1 atm (10.1 kPa) of hydrogen could be reversed completely when hydrogen was removed from the coculture (2). However, the coculture needed a lag period of 4 to 5 days before butyrate consumption was restored, even though the concentration of the dissolved hydrogen remaining in the cocultures was equal to that normally found during exponential growth of the coculture. The addition of 81.4 mM acetate was required for total inhibition of butyrate consumption in the thermophilic butyrate-degrading coculture. This is in accordance with a concentration of 80 mM acetate that has been found to inhibit propionate oxidation in a mesophilic sludge digestor (13). However, unlike these experiments, lower concentrations of acetate also had an inhibitory effect on the thermophilic butyrate-degrading coculture. Boone and Xun (6) found a slight inhibition when 20 mM acetate was added to a mesophilic propionate-degrading enrichment culture, but the influence on the propionate degradation rate was not reported. The higher specific butyrate consumption rates found in the thermophilic triculture can partly be explained as a consequence of acetate utilization and prevention of product inhibition by acetate. The experiments with acetate additions showed a tendency for a higher inhibition 24 h after the addition of acetate, which could be a result of the increase in the acetate concentration resulting from the degradation of butyrate. As previously demonstrated for the inhibition of the coculture with hydrogen, the inhibitory effect of acetate could be reversed when the acetate was metabolized by the acetate-utilizing methanogens in the triculture; no lag period occurred prior to butyrate utilization. No further degradation of butyrate was observed in cocultures that were totally inhibited with acetate (with 100 mM acetate added) even after an extended incubation time.

In summary, product inhibition by hydrogen may be an important factor in anaerobic digestion; only a small increase in the partial pressure of hydrogen above the normal level inhibits the metabolism of the fatty acid-oxidizing acetogenic bacteria (13; this study). Simultaneously with the suppression of acetogenic activity, an increase in the hydrogen partial pressure leads to a shift in the fermentation products of the fermentative bacteria toward the formation of fatty acids such as propionate and butyrate. This results in a drop in pH and finally to digestor failure. Acetate has also been found to inhibit the fatty acid-oxidizing acetogenic bacteria, but much higher concentrations that the normal in situ concentrations of 0.2 to 0.3 mM found in digestors (1, 12) are needed for significant inhibition (13; this study). Normally, the shift in the internal metabolism of the fermentative bacteria accompanying a mild imbalance in the anaerobic digestor provides the aceticlastic methanogens with sufficient time to metabolize the overproduction of acetate. However, in a situation in which the aceticlastic methanogens are selectively inhibited, the concentration of acetate can rise to inhibitory levels. The results obtained with the thermophilic butyrate-degrading bacterium indicate that product inhibition with acetate can be of importance when fatty acid-oxidizing bacteria are cultivated in cocultures with only a hydrogen-oxidizing methanogen as a sole partner, as has been the case for most of the studies described previously (5, 10, 19, 20). This could also be one of the reasons for the differences observed between the growth rates of mesophilic enrichment cultures on butyrate and propionate (0.015 and 0.017 h^{-1}, respectively) (15) and the growth rates determined with cocultures of butyrate- and propionate-degrading bacteria together with hydrogen-oxidizing methanogens (0.00825 and 0.004 h^{-1}, respectively).

The results provided here indicate that greater attention should be paid to the elimination of both hydrogen and acetate in future work with fatty acid-oxidizing acetogenic bacteria.

ACKNOWLEDGMENT

We thank Karin Vestberg and Anne Breum for excellent technical assistance and Robert A. Mah for critical review of the manuscript.

This study was supported by grant 5.17.4.6.17 from the Danish Technical Research Council and a grant from the Nordic Ministery Council.

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