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Inhibition of the Fermentation of Propionate to Methane by Hydrogen, Acetate, and Propionate

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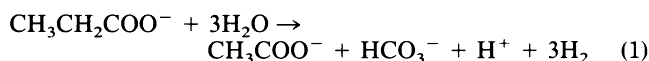
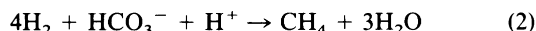
Inhibition of the fermentation of propionate to methane and carbon dioxide by hydrogen, acetate, and propionate was analyzed with a mesophilic propionate-acclimatized sludge that consisted of numerous flocs (size, 150 to 300 μm). The acclimatized sludge could convert propionate to methane and carbon dioxide stoichiometrically without accumulating hydrogen and acetate in a propionate-minimal medium. Inhibition of propionate utilization by propionate could be analyzed by a second-order substrate inhibition model (shown below) given that the substrate saturation constant, K_s, was 15.9 μM; the substrate inhibition constant, K_i, was 0.79 mM; and the maximum specific rate of propionate utilization, q_m, was 2.15 mmol/g of mixed-liquor volatile suspended solids (MLVSS) per day: $q_s = q_m S/[K_s + S + (S^2/K_i)]$, where q_s is the specific rate of propionate utilization and S is the initial concentration of undissociated propionic acid. For inhibition by hydrogen and acetate to propionate utilization, a noncompetitive product inhibition model was used: $q_s = q_m/[1 + (P/K_p)^n]$, where P is the initial concentration of hydrogen or undissociated acetic acid and K_p is the inhibition constant. Kinetic analysis gave, for hydrogen inhibition, $K_{p(H_2)} = 0.11$ atm (= 11.1 kPa, 71.5 μM), $q_m = 2.40$ mmol/g of MLVSS per day, and $n = 1.51$ and, for acetate inhibition, $K_{p(HAc)} = 48.6$ μM, $q_m = 1.85$ mmol/g of MLVSS per day, and $n = 0.96$. It could be concluded that the increase in undissociated propionic acid concentration was a key factor in inhibition of propionate utilization and that hydrogen and acetate cooperatively inhibited propionate degradation, suggesting that hydrogenotrophic and acetoclastic methanogens might play an important role in enhancing propionate degradation to methane and carbon dioxide.

Production of CH_4 from propionate is an important intermediate step in the bioconversion of organic matter to CH_4 and CO_2. Propionate degradation requires obligate proton-reducing acetogens that form acetate, H_2, and CO_2 from propionate and H_2- and acetate-utilizing methanogens (2, 4, 13). The equations for the reactions of the respective bacterial groups are as follows:



$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2 \quad (1)$$

$$\Delta G_0' = +76.1 \text{ kJ/reaction}$$



$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \quad (2)$$

$$\Delta G_0' = -135.6 \text{ kJ/reaction}$$

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad (3)$$

$$\Delta G_0' = -31.0 \text{ kJ/reaction}$$

Reaction 1 is inhibited by the accumulation of H_2 (2, 4, 17). The partial pressure of H_2 must be kept below 10^{-6} to 10^{-4} atm (0.1 to 10.1 Pa) in order for reaction 1 to proceed (10). This is generally accomplished by the reduction of CO_2 to CH_4 by H_2-using methanogens (5, 6, 16, 23). If this reaction is inhibited, i.e., when organic matter is completely converted to CH_4 and CO_2, it would be expected that propionate and perhaps acetate would accumulate. These compounds in turn may further inhibit the conversion of propionate to CH_4 and CO_2. Although acetate is a product of reaction 1, the influence of acetate on the reaction has not been fully investigated (9, 26).

In the present study, we investigated the influence of acetate, propionate, and H_2 concentrations on propionate degradation. We used an acclimatized culture derived from an anaerobic digestion sludge. This acclimatized sludge converted propionate to CH_4 and CO_2 under mesophilic conditions. Propionate degradation was inhibited by propionate, acetate, and H_2. The kinetics of inhibition by these compounds were analyzed by a second-order substrate and a noncompetitive inhibition model. Inhibition by propionate and acetate was influenced by pH in a manner that suggests that the inhibition was due to the free acids rather than the acid anions.

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MATERIALS AND METHODS

Microorganisms. The granulated sludge (mesophilic) of anaerobic digestion of starch wastewater was supplied by the Biotechnology Research Laboratory, Kobe Steel Ltd., Kobe, Japan. An H_2-using methanogen isolated from the above sludge was also used. The methanogen was a gram-positive, long, rod-shaped bacterium and could utilize hydrogen-carbon dioxide and formate but not methanol, methylamines, and acetate as growth substrates.

Media. A sulfate-free minimal medium composed of inorganic salts, including trace elements, a vitamin mixture, and cysteine as the sole sulfur source, was used. The medium was the same as that used for *Methanosarcina barkeri* (15) except that sodium propionate was added instead of methanol. For culturing H_2-using methanogens, the same medium was used without propionate or methanol. It was supplemented with Na_2CO_3 (2.48 g/liter) and H_2-CO_2 (80:20, vol/vol) at 2.5 atm (253 kPa).

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Acclimatization. To acclimatize the digestor sludge to the propionate-minimal medium, the original granulated sludge was centrifuged (3,700 × *g*, 20 min) and the collected sludge was suspended (2.5 g of mixed-liquor volatile suspended solids [MLVSS] per liter) in 500 ml of the minimal medium containing 20 mM propionate into a 700-ml serum bottle whose headspace was filled with N_2 gas (99.9999%, vol/vol). The culture was incubated statically at 37°C. Production of CH_4 was monitored regularly, and when gas evolution ceased, e.g., after 2 weeks, the culture broth was centrifuged (3,700 × *g*, 20 min). At this point, the collected sludge was suspended in the same minimal medium mentioned above and cultivated statically for 2 weeks to maintain the acclimatized culture. After repeated batch cultures for 4 months, 20 mM propionate was completely consumed in 1 week without accumulation of acetate or H_2. A modified Hungate technique (3, 19) was used throughout to maintain anaerobic conditions.

Kinetics of propionate consumption. About 200 ml of the acclimatized culture was centrifuged (930 × *g*, 10 min). The suspended solid collected was suspended in minimal medium without propionate in the range of 4 to 40 g of MLVSS per liter and incubated statically at 37°C for 6 h. A 5-ml portion of the suspended medium was inoculated into 125-ml serum vials with 45 ml of the propionate-minimal medium for all kinetic studies. Cultures were incubated at 37°C with shaking (100 rpm). To determine the effect of initial propionate concentration and pH on propionate utilization, initial propionate concentrations of 6 to 85 mM were used over a pH range of 6.0 to 7.6. These pH values were adjusted by adding 1 N HCl or NaOH before inoculation. During the culture, propionate concentrations were measured up to 75 h after incubation, and propionate utilization rate was calculated from a linear disappearance of propionate which could be observed for 36 to 75 h after incubation under each condition. The effect of H_2 on propionate utilization rate was determined by adding 0.01 to 0.91 atm of H_2 (1.01 to 92.2 kPa) to 69-h-old cultures that still contained 15 mM propionate. A linear disappearance of propionate for 26 h after H_2 injection was used for calculation of the propionate utilization rate. The rates of propionate (12 mM) utilization in the presence of initial acetate concentrations of 2.5 to 300 mM (added as sodium acetate) at pH levels of 6.5 and 7.2 were calculated from the linear disappearance of propionate for 69 h after incubation. The rate of propionate utilization obtained was divided by the respective initial MLVSS concentration to express the specific rate of propionate utilization.

Determination of kinetic constants. A second-order substrate inhibition model (see Results, equation 4) and a noncompetitive product inhibition model (see Results, equation 5) were used to estimate kinetic constants. The data fitting of specific rates of propionate utilization to equations 4 and 5 was computed statistically by using the nonlinear least-squares regression method.

Microscopic observation. During propionate fermentation to CH_4 and CO_2, the freely dispersed and granulated bacteria were observed microscopically (Nikon microscope L-I).

Analyses. The volumes of evolved CH_4 and CO_2 were determined by displacing a saturated NaCl solution. Suspended solid material in the cultures was recovered by filtration (0.45-μm pore size; Millipore Corp.). The filters were dried at 105°C for 5 h, and the weight of the mixed-liquor suspended solids was determined. The filters were ashed by burning in a crucible to constant weight, and the weight of the ash was determined. Thus, the MLVSS was calculated as the difference between the weights of mixed-liquor suspended solids and ash. The dry cell concentration of the H_2-using methanogen culture was assessed from the whole-cell protein content (15). Propionate and acetate were determined with a gas chromatograph (GC-8AG; Shimadzu) equipped with a polyethylene glycol 6000 (30/60-mesh) column and a flame ionization detector. The column temperature was 170°C, and the injector and flame ionization detector temperature was 200°C. Methane, carbon dioxide, and hydrogen were determined with a gas chromatograph equipped with a thermal conductivity detector (21).

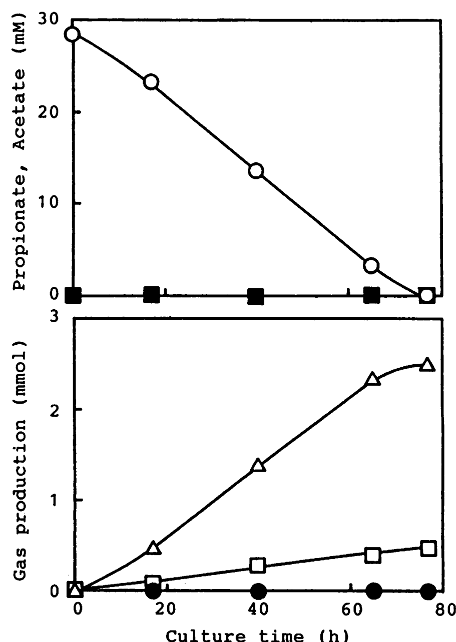

FIG. 1. Methanogenic fermentation of propionate by a propionate-acclimatized sludge in a propionate-minimal medium. Culture inoculum size, 10% (vol/vol), was equivalent to 4.23 g of MLVSS per liter. Culture was incubated in a 125-ml serum vial (medium, 50 ml; initial pH, 7.0; headspace, initially N_2) at 37°C with shaking (100 rpm). Symbols: ○, propionate; ■, acetate; △, methane; □, carbon dioxide; ●, hydrogen.

Chemicals. All chemicals were of reagent grade purchased from commercial sources. N_2 and H_2 of >99.9999% (vol/vol) purity (Chugoku Teisan, Hiroshima, Japan) were used without any treatments.

RESULTS

Propionate conversion to methane. A 5-ml portion of the dense propionate-acclimatized sludge (42.3 g of MLVSS per liter) harvested by centrifugation was inoculated into a 125-ml serum vial with 45 ml of minimal medium. The time course of propionate disappearance and gas formation is shown in Fig. 1. The overall ratio of CH_4 produced to propionate consumed was 1.74. The expected ratio for complete bioconversion of propionate to CH_4 and CO_2 is 1.75. H_2 or acetate was not detected. The majority of organisms were present in the form of 150- to 300-μm flocs that contained rods, sarcina, and *Methanothrix*-like bacteria.

Inhibition by propionate. Propionate disappearance was measured with different initial concentrations of propionate at different initial pH values. The relationship between the rate of propionate disappearance and initial propionate concentration was analyzed by using an equation for a second-order substrate inhibition model (8, 22, 24):

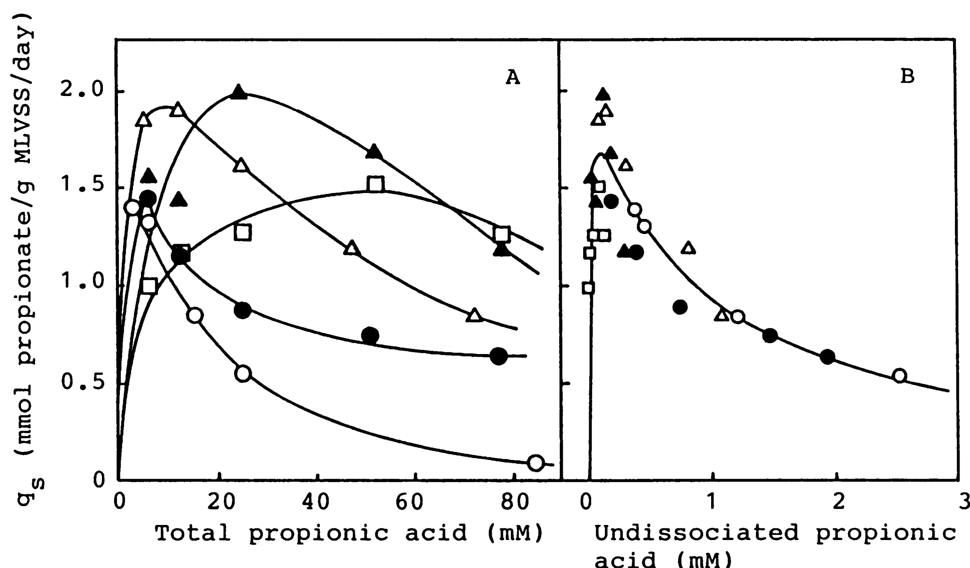

FIG. 2. Propionate inhibition against specific rate of propionate utilization; q_s as a function of initial pH in methanogenic fermentation of propionate. (A) q_s versus initial concentration of total propionic acid; (B) q_s versus initial concentration of undissociated propionic acid (calculated by pK_a = 4.89; 37°C). q_s values were calculated from linear disappearance of propionate at 36, 47, or 75 h, respectively. Initial cell concentration was 0.437 g of MLVSS per liter. Solid line in Fig. 2B was computed by using equation 4 (see text). For cultural conditions, see legend to Fig. 1. Symbols (initial pH): ○, 6.0; ●, 6.4; △, 6.8; ▲, 7.3; □, 7.6.

$$q_s = \frac{q_m S}{K_s + S + S^2/K_i} \quad (4)$$

where q_s is the specific rate of propionate utilization, millimoles per gram of MLVSS per day; q_m is maximum value of q_s; S is initial concentration of undissociated propionic acid, millimolar; K_s is substrate saturation constant, millimolar; and K_i is substrate inhibition constant, millimolar.

The rate of disappearance decreased with increasing concentrations of propionate (Fig. 2A). Maximum rates were obtained at pH 6.8 and 7.3, with marked inhibition observed at pH 6.0 and 6.4. This indicated that the inhibition was due to undissociated propionic acid. The rates of propionate utilization as a function of the concentration of undissociated acid (pK_a = 4.89, 37°C) are shown in Fig. 2B. The solid line was derived by computer analysis, using a nonlinear regression method and equation 4. The kinetic constants of equation 4 derived from a data-fitting procedure were as follows: K_s = 15.9 μM; K_i = 0.79 mM; and q_m = 2.15 mmol/g of MLVSS per day.

H_2 inhibition. When H_2 was added to 69-h cultures, propionate degradation was inhibited (Fig. 3A). The extent of the inhibition depended on the partial pressure of H_2. A noncompetitive inhibition equation was used to analyze the relationship between the rate of propionate utilization and the partial pressure of H_2 (14, 20, 22, 25):

$$q_s = \frac{q_m}{1 + (P/K_p)^n} \quad (5)$$

where P is the partial pressure of H_2 when added to a 69-h culture, atmospheres; K_p is the inhibition constant, atmospheres; and n is the exponent of inhibition.

The results of the analysis are shown in Fig. 3B. The solid line was derived by computer analysis, using a nonlinear regression method and equation 5. The constants derived from data fitting were as follows: $K_{p(H_2)}$ = 0.11 atm (11.1 kPa; 71.5 μM); q_m = 2.40 mmol/g of MLVSS per day; and n = 1.51.

An H_2-using methanogen added to a culture inhibited by 0.91 atm of H_2 (92.2 kPa) reversed the inhibition (Fig. 4). This suggests that inhibition of the conversion of propionate to acetate, CO_2, and H_2 by H_2 was reversed by consumption of H_2 by the added methanogen.

Acetate inhibition. Acetate inhibition of propionate utilization was examined by adding 2.5 to 300 mM acetate to cultures. The specific rates of propionate utilization (q_s) were calculated from the decrease in propionate at 69 h. Propionate disappearance was linear over this period of incubation. Inhibition by acetate was greater at pH 6.5 than at pH 7.2 (Fig. 5A). The data for q_s were replotted against undissociated acetic acid concentrations, which were calculated by using a pK_a of 4.76 at 37°C (Fig. 5B). The solid line was derived by computer analysis, using a nonlinear regression method and equation 5. The constants derived from data fitting were as follows: $K_{p(HAc)}$ = 48.6 μM; q_m = 1.85 mmol/g of MLVSS per day; and n = 0.96.

DISCUSSION

Inhibition of propionate degradation by propionate and acetate was probably due to the undissociated acid forms of these compounds (Fig. 2B and 5B). The increase in concentration of undissociated acids may accelerate their entry into cells and cause a drop in intracellular pH due to exclusion of the anions, leaving behind the protons. The excess protons would have to be extruded out to maintain a functional proton gradient. The process of proton extrusion from the cell would require hydrolysis of ATP, meaning a decrease in ATP availability for growth and metabolism of the organism. Consequently, the acids might act as uncouplers of the membrane proton gradient (11). In connection with this, Menzel and Gottschalk (18) measured the intracellular pH in growing *Acetobacterium wieringae* and *Acetobacter aceti* and found a decrease in intracellular pH with an increase in extracellular acetic acid. They demonstrated that neither the anaerobic nor the aerobic acetogen was able to maintain a

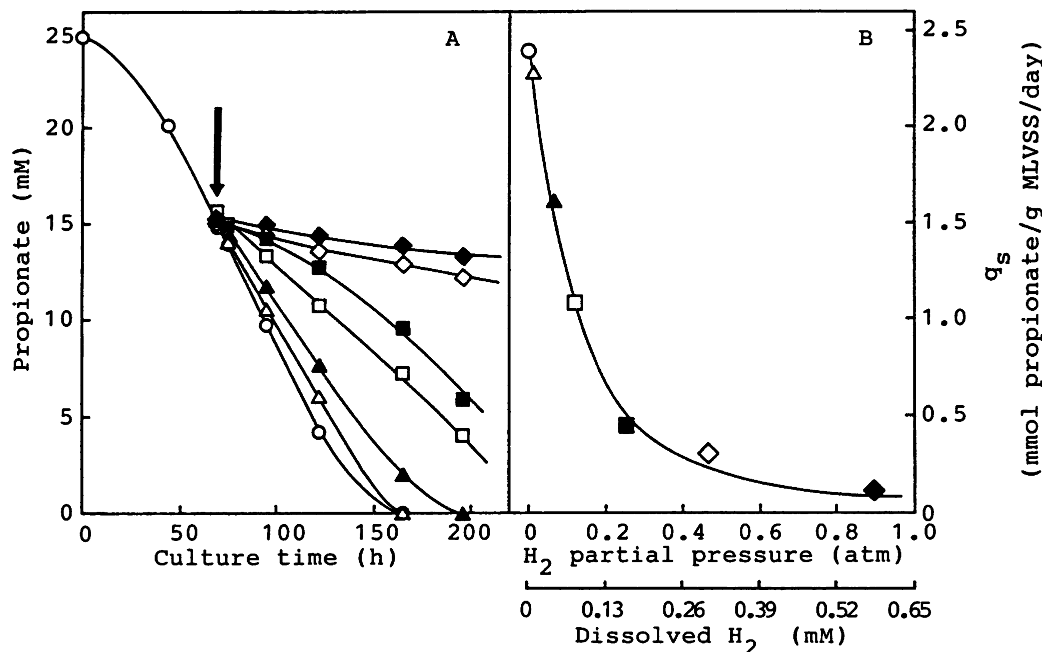

FIG. 3. H_2 inhibition of propionate utilization in methanogenic fermentation of propionate. (A) Arrow, H_2 injection into headspace of vial; (B) q_s versus initial H_2 partial pressure. q_s values were calculated from linear disappearance of propionate for 26 h after H_2 injection. Initial cell concentration was 2.15 g of MLVSS per liter. The dissolved H_2 concentration was calculated by Bunsen coefficient at 37°C. Solid line in Fig. 3B was computed by using equation 5 (see text). For cutural conditions, see legend to Fig. 1. Symbols (H_2, atm): ○, control; △, 0.01 (1 kPa); ▲, 0.07 (7.1 kPa); □, 0.12 (12.2 kPa); ■, 0.25 (25.3 kPa); ◇, 0.47 (47.6 kPa); ◆, 0.91 (92.2 kPa).

large ΔpH in the presence of high concentrations of acetic acid, which acts as a protonophore at low external pH levels.

Heyes and Hall (12) divided propionate-consuming bacteria from anaerobic digestors into two subgroups based on the K_s for propionate. One group had a K_s of 0.15 mM (pH 7.0; μ = 0.0054 h^{-1}); the other had a K_s of 4.45 mM (pH 7.0; μ = 0.05 h^{-1}). K_s values between 0.04 and 0.19 mM (pH 7.0) were observed with mixed sludge from a waste treatment plant (13), and K_s values of 0.82 to 1.36 mM (pH 7.0) were observed with granulated sludge (7). In the present experiments, the apparent K_s was 2.0 mM at pH 7.0, which corresponds to 15.9 μM for undissociated propionic acid.

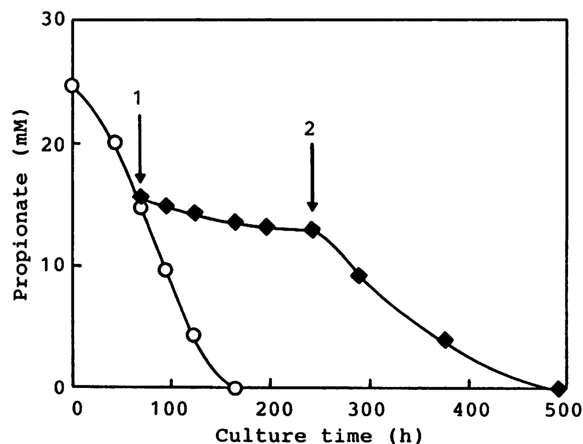

FIG. 4. Acceleration of propionate consumption by injection of H_2-utilizing methanogen cells (corresponds to 0.91 atm of H_2 in Fig. 3A). Arrow 1, H_2 injection (0.91 atm) at 69 h; arrow 2, injection of methanogen cells (0.35 g of dry cells per liter) at 241 h. Symbols: ○, control; ◆, 0.91 atm (92.2 kPa) of H_2.

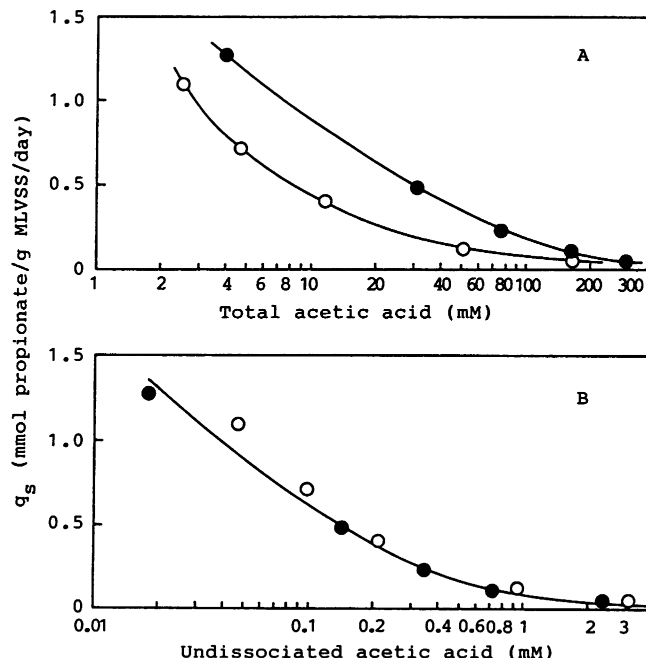

FIG. 5. Acetate inhibition of propionate utilization in methanogenic fermentation of propionate. (A) q_s versus initial concentration of total acetic acid; (B) q_s versus initial concentration of undissociated acetic acid (calculated by pK_a = 4.76; 37°C). Medium contained 12 mM propionate and an initial cell concentration of 0.224 g of MLVSS per liter. q_s values were calculated from the linear decrease in propionate for 69-h cultures. Solid line in Fig. 5B was computed by using equation 5 (see text). For cultural conditions, see legend to Fig. 1. Symbols (initial pH): ●, 7.2; ○, 6.5.

Granulated bacteria appear to have higher K_s values (between 1 and 4 mM).

The production of H_2 from oxidation of volatile fatty acids is considered to be feasible only at partial pressures of H_2 of 10^{-6} to 10^{-4} atm (0.1 to 10.1 Pa). However, in the present experiments, propionate utilization was not significantly inhibited at 7×10^{-2} atm (7.1 kPa). The apparent $K_{p(H_2)}$, calculated by the initial partial pressure of H_2, was 0.11 atm (11.1 kPa; 71.5 μM). Bacteria inside the flocs might be partially protected from exogenous H_2 by adjacent hydrogenotrophic methanogens. The removal of H_2 by added methanogens relieved the inhibition of propionate degradation by H_2, as was the case for butyrate degradation (1).

Acetate inhibition of propionate utilization has been noted by others. A 60% decrease of propionate consumption was obtained with methanogenic sludge with 3.3 mM acetate at pH 7.0 (9), and up to 50% inhibition was obtained with 15 mM acetate at pH 7.0 (26). The apparent K_p of 48.6 μM for undissociated acetic acid obtained in our study corresponds to 8.3 mM total acetic acid at pH 7.0.

It is of interest to note that the inhibition constant (K_p) for acetate of 48.6 μM was the same order of magnitude as the constant for H_2, which was 71.5 μM (dissolved hydrogen). This suggests that the removal of both H_2 and acetate by methanogens is important for maintaining maximal rates of propionate degradation.

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