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Enrichment of Thermophilic Propionate-Oxidizing Bacteria in Syntrophy with *Methanobacterium thermoautotrophicum* or *Methanobacterium thermoformicicum*

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Thermophilic propionate-oxidizing, proton-reducing bacteria were enriched from the granular methanogenic sludge of a bench-scale upflow anaerobic sludge bed reactor operated at 55°C with a mixture of volatile fatty acids as feed. Thermophilic hydrogenotrophic methanogens had a high decay rate. Therefore, stable, thermophilic propionate-oxidizing cultures could not be obtained by using the usual enrichment procedures. Stable and reproducible cultivation was possible by enrichment in hydrogen-pregrown cultures of *Methanobacterium thermoautotrophicum* ΔH which were embedded in precipitates of FeS, achieved by addition of $FeCl_2$ to the media. The propionate-oxidizing bacteria formed spores which resisted pasteurization for 30 min at 90°C or 10 min at 100°C. Highly purified cultures were obtained with either *M. thermoautotrophicum* ΔH or *Methanobacterium thermoformicicum* Z245 as the syntrophic partner organism. The optimum temperature for the two cultures was 55°C. Maximum specific growth rates of cultures with *M. thermoautotrophicum* ΔH were somewhat lower than those of cultures with *M. thermoformicicum* Z245 (0.15 and 0.19 day^{-1}, respectively). Growth rates were even higher (0.32 day^{-1}) when aceticlastic methanogens were present as well. *M. thermoautotrophicum* ΔH is an obligately hydrogen-utilizing methanogen, showing that interspecies hydrogen transfer is the mechanism by which reducing equivalents are channelled from the acetogens to this methanogen. Boundaries of hydrogen partial pressures at which propionate oxidation occurred were between 6 and 34 Pa. Formate had a strong inhibitory effect on propionate oxidation in cultures with *M. thermoautotrophicum*. Inhibition by formate was neutralized by addition of the formate-utilizing methanogen or by addition of fumarate. Results indicate that formate inhibited succinate oxidation to fumarate, an intermediate step in the biochemical pathway of propionate oxidation.

Anaerobic conversion of organic matter leads to the intermediate formation of fatty acids (6, 22, 29, 35). Under methanogenic conditions, fatty acids with more than two carbon atoms are degraded by syntrophic consortia of different physiological types of bacteria (6, 11, 13, 29). Three different types of bacteria are needed for complete conversion of these compounds to methane and carbon dioxide. Acetogenic bacteria oxidize higher fatty acids to acetate and hydrogen (2, 7, 11, 13, 14) or, as proposed recently, to acetate and formate (8, 32). Gibbs free energy changes ($\Delta G'$) of reactions involved in the oxidation of butyrate and propionate coupled to hydrogen or formate formation are given in Table 1. Because of unfavorable energetics, oxidation of propionate and butyrate is possible only when products are removed efficiently by methanogens. This results in an obligate interdependency of acetogenic and methanogenic bacteria. Some fatty acid-oxidizing cultures have been described previously (2, 3, 7, 9, 14, 21, 23, 24, 26–28, 30). Mesophilic and thermophilic butyrate-oxidizing bacteria were obtained in defined cocultures with methanogens (2, 3, 14, 23, 24, 28, 30), and in some cases the butyrate-degrading bacterium was obtained in pure culture (4, 5, 20). Up to now, only mesophilic propionate-oxidizing cultures have been described (7, 9, 15, 21, 26, 27). *Syntrophobacter wolinii* is the only syntrophic propionate oxidizer which is maintained in a defined coculture with a *Desulfovibrio* sp. (7). Stable, thermophilic, syntrophic, propionate-oxidizing cultures have not been described thus far. In the present paper, we describe the enrichment of thermophilic, spore-forming, propionate-oxidizing bacteria in syntrophy with *Methanobacterium thermoautotrophicum* ΔH or *Methanobacterium thermoformicicum* Z245.

MATERIALS AND METHODS

Organisms. *M. thermoautotrophicum* ΔH (DSM 1053) was kindly provided by G. D. Vogels (University of Nijmegen, Nijmegen, The Netherlands). *M. thermoformicicum* Z245 (DSM 3720) was a gift of J. P. Touzel (INRA, Villeneuve d'Ascq, France). *Methanospirillum hungatii* DSM 864 was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). *Methanothrix* sp. strain CALS-1 (DSM 3870) was a gift of S. H. Zinder (Cornell University, Ithaca, N.Y.). Propionate-oxidizing bacteria and aceticlastic methanogens enriched from thermophilic methanogenic granular sludge are described below.

Media and cultivation. A bicarbonate-buffered medium containing (in grams per liter): $Na_2HPO_4 \cdot 2H_2O$ (0.53), KH_2PO_4 (0.41), NH_4Cl (0.3), $CaCl_2 \cdot 2H_2O$ (0.11), $MgCl_2 \cdot 6H_2O$ (0.10), $NaHCO_3$ (4.0), and $Na_2S \cdot 9H_2O$ (0.48) as well as acid and alkaline trace elements (1 ml of each per liter) and vitamins (0.2 ml/liter) was used. The acid trace elements solution contained the following (millimolar): $FeCl_2$, 7.5; H_3BO_4, 1; $ZnCl_2$, 0.5; $CuCl_2$, 0.1; $MnCl_2$, 0.5; $CoCl_2$, 0.5; $NiCl_2$, 0.1; and HCl, 50. The alkaline trace elements solution

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TABLE 1. Reactions involved in propionate and butyrate oxidation in methanogenic ecosystems[a]

Reaction	$\Delta G^{\circ\prime}$ (kJ) at 25°C	$\Delta G'$ (kJ) at 55°C
$Butyrate^- + 2H_2O \rightarrow 2\ Acetate^- + 2H_2 + H^+$	+48.1	+37.9
$Butyrate^- + 2HCO_3^- \rightarrow 2\ Acetate^- + 2\ Formate^- + H^+$	+45.5	+36.1
$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + 3H_2 + H^+$	+76.1	+62.3
$Propionate^- + 2HCO_3^- \rightarrow Acetate^- + 3\ Formate^- + H^+$	+72.2	+59.7
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	−31.0	−34.7
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + H_2O$	−135.6	−122.5
$4\ Formate^- + H_2O + H^+ \rightarrow CH_4 + 3HCO_3^-$	−130.4	−118.9

[a] Gibbs free energy changes at 25°C were taken from the review of Thauer et al. (31). Gibbs free energy changes at 55°C were calculated by using the van't Hoff equation, standard enthalpy values of compounds (10), and Gibbs free energy changes at 25°C. $\Delta G^{\circ\prime}$ is ΔG° at pH 7.

was composed of the following (millimolar): Na_2SeO_3, 0.1; Na_2WO_4, 0.1; Na_2MoO_4, 0.1; and NaOH, 10. The following vitamins had the indicated compositions (in grams per liter): biotin, 0.02; niacin, 0.2; pyridoxine, 0.5; riboflavin, 0.1; thiamine, 0.2; cyanocobalamine, 0.1; *p*-aminobenzoic acid, 0.1; and pantothenic acid, 0.1. Vitamins were filter sterilized; other compounds were sterilized by heat. Unless stated otherwise, all incubations were done at 55°C in 120-ml serum bottles with 50 ml of medium. For cultivation of methanogens, a gas phase of 172-kPa (1.7-atm) H_2-CO_2 (80:20, vol/vol) was used. After inoculation (1% from stock cultures), bottles were incubated while being shaken at 400 rpm. Propionate-oxidizing bacteria were routinely grown in hydrogen-pregrown cultures of *M. thermoautotrophicum* ΔH. After growth of the methanogen on H_2-CO_2, the gas phase was changed to 172-kPa N_2-CO_2 (80:20, vol/vol), and $FeCl_2$ was added to a final concentration of 1 mM. Propionate was added from a 1 M stock solution to give a final concentration of 20 mM. Routinely, dense inocula (an inoculum size of 5 or 10%) of propionate-oxidizing cultures were used, and bottles were incubated while stationary. Aceticlastic methanogens which were used in this study were enriched by repeated transfer (10%) in media with 20 mM sodium acetate as the sole energy and carbon source. In experiments in which combinations of hydrogenotrophic and aceticlastic methanogens were used, acetate-grown and hydrogen-grown cultures were mixed in a ratio of 1 to 1 or mixed in the same ratio with sterile medium. After addition of propionate and $FeCl_2$, a propionate-oxidizing culture was inoculated (2%) and incubated while shaken or stationary. At various times, samples were taken aseptically by syringe.

Starvation of methanogens. The decay rate of hydrogenotrophic methanogens was estimated from the rate of methane formation of cultures which were starved for different periods. Hydrogen-carbon dioxide-grown cultures were stored at 55°C. After various periods, the gas phase of two or three bottles was changed to 172-kPa H_2-CO_2 (80:20), and methane formation was monitored in time. Rates were calculated from methane formation between 1 and 5 h of incubation. The decay rate was calculated from the decrease of the rate of methanogenesis upon storage at 55°C.

Analytical methods. Organic acids were determined by high-performance liquid chromatography (LKB) with a Chrompack organic acid column (temperature, 60°C) at a flow rate of 0.60 ml/min with 0.01 N H_2SO_4 as the eluent. Fatty acids were also analyzed by gas chromatography with a Chrompack (CP 9000) gas chromatograph equipped with a Chromosorb 101 column (80/100 mesh, 2 m by 2 mm). The column temperature was 160°C, the injection port temperature was 220°C, and the flame ionization detector temperature was 240°C. The carrier gas (30 ml/min) was nitrogen saturated with formic acid. Gases were determined by gas chromatography with a Packard-Becker 417 gas chromatograph equipped with a thermal conductivity detector and molecular sieve. The column temperature was 50°C, and the carrier gas was argon at a flow rate of 20 ml/min.

Miscellaneous methods. Cultures were pasteurized in 18-ml Hungate tubes containing 10 ml of a spore-forming culture and a gas phase of 172-kPa N_2-CO_2. Bacteria were counted with a Bürker-Bürk counting chamber. Specific growth rates of propionate-oxidizing bacteria were determined from the rates of methane formation and propionate degradation, in case acetate-degrading bacteria were present in the cultures, and from the rates of acetate formation for cultures in which acetate was not degraded.

RESULTS

Enrichment. Thermophilic methanogenic sludge of a bench-scale upflow anaerobic sludge bed reactor was used for the enrichment of thermophilic propionate-oxidizing bacteria. The reactor was inoculated with mesophilic granular sludge from a potato-processing factory (Aviko, Steenderen, The Netherlands) and operated for 3 months at 55°C with a mineral salt solution containing a fatty acid mixture as the substrate and yeast extract (about 0.05 g/liter) as the source of vitamins. The average influent concentrations of acetate, propionate, and butyrate were 140, 27, and 19 mM, respectively. The design and operation of the reactor have been described elsewhere (33). In the reactor, a propionate removal efficiency of about 90% was achieved. For enrichment of propionate-oxidizing bacteria, granules were crushed in an anaerobic glove box under an atmosphere of N_2-H_2 (96:4) and inoculated in media with propionate as the sole carbon and energy source. During incubation at 55°C, small gas bubbles were released from the sediment, indicating that a major part of the methanogenic activity was associated with immobilized biomass. Transfer of the sediment layer into fresh media resulted in fast continuation of methane production. Only slow or, in some cases, even no methane production was found when suspended cells were transferred. Because close contact between acetogenic and methanogenic bacteria is essential for an efficient interspecies electron transfer, we created artificial sediments in the cultures. The addition of $FeCl_2$, which led to the formation of FeS precipitates, appeared to be very effective in this respect. When $FeCl_2$ was added to pure cultures of *M. thermoautotrophicum* ΔH or *M. thermoformicicum* Z245, more than 99% of the initially suspended bacteria were embedded in the precipitate. Complete settlement of the FeS and cells generally took about 10 h at 55°C. By adding $FeCl_2$ to media and by using dense inocula (5 to 10%), stable propionate-oxidizing cultures were obtained. Figure 1 shows results for propionate conversion by such enrichment cultures at 55°C. The apparent doubling time of propionate oxidizers as estimated from the rate of propionate oxidation or methane production was 65 to 70 h ($\mu = \pm 0.25\ day^{-1}$). Remarkably,

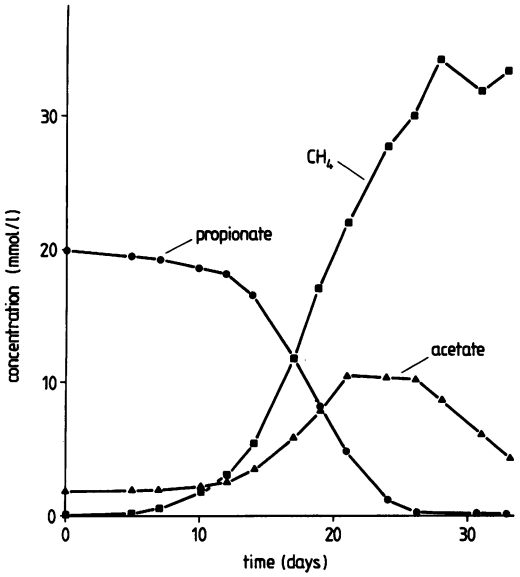

FIG. 1. Propionate oxidation by a thermophilic enrichment culture. Incubation was done at 55°C, and 1 mM $FeCl_2$ was added to form a precipitate of FeS. During incubation, active methanogenesis occurred within the precipitate.

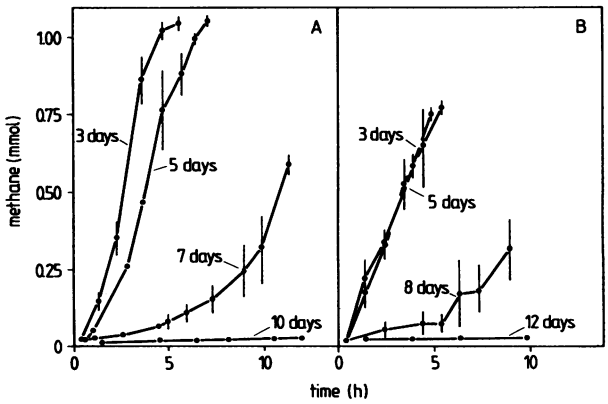

FIG. 2. Methane formation by starved cells of *M. thermoautotrophicum* ΔH (A) and *M. thermoformicicum* Z245 (B). Indicated days refer to the period of incubation at 55°C after hydrogen had been depleted. Methane is expressed as millimoles per vial.

although methanogens were entirely embedded in the sediment, propionate-oxidizing bacteria were not. Upon incubation of the cultures, the medium above the FeS layer became turbid. Transfer of these suspended bacteria to FeS-embedded cultures of *M. thermoautotrophicum* ΔH led to a fast continuation of propionate degradation. To obtain defined methanogenic cocultures, dilutions in fresh media had to be made. Results of a typical experiment in which a propionate-oxidizing culture was diluted in fresh media and in hydrogen-pregrown cultures of *M. thermoautotrophicum* ΔH are given in Table 2. In the absence of methanogens, clear growth occurred only at an inoculum size of 5%, whereas growth was observed up to a dilution of 625×10^{-8} when methanogens were present. In the cultures with the methanogens, acetate did not accumulate, while in the cultures without methanogens, acetate remained present. Control experiments showed that the methanogens alone did not degrade measurable amounts of propionate or acetate.

Stability of methanogens. Although the presumable propionate-oxidizing bacterium after growth in media with 20 mM propionate as the substrate was present in numbers of about 10^8/ml, we never obtained growth at dilutions higher than 10^{-6} when the bacterium was transferred into hydrogen-pregrown cultures of methanogens. In addition, attempts to achieve colony formation in roll tubes or in agar shakes in which hydrogen-pregrown methanogens were present were not successful. Because growth of thermophilic propionate oxidizers is slow and because it is known that thermophilic methanogenic bacteria cannot be stored at their growth temperatures after substrate depletion, we tried to estimate decay rates of *M. thermoautotrophicum* ΔH and *M. thermoformicicum* Z245 at 55°C. Figure 2A shows methane production by cultures of *M. thermoautotrophicum* ΔH which had been starved for different periods at 55°C. Assuming that the methane production rate per living cell is constant, a decay rate of about 0.48 day^{-1} was calculated. When methanogenic cultures were stored unfed at 55°C for 2 weeks or longer, methane formation did not occur after the addition of hydrogen, indicating that the cultures were dead. Methanogenic cells were more stable when embedded in the FeS precipitate. In that case, the decay rate was about 0.29 day^{-1}, and some of the bottles started to form methane even after storage for 4 weeks at 55°C (results not shown). A decay rate of about 0.48 day^{-1} was also determined for suspended cells of *M. thermoformicicum* Z245 (Fig. 2B). However, viable cells were still present after 4 weeks of incubation, even in the absence of FeS. These results show that *M. thermoautotrophicum* is more sensitive to starvation than *M. thermoformicicum*, but for both strains, the specific decay rates in the absence of substrate were in the same range as the specific growth rates of propionate oxidizers.

TABLE 2. Effect of addition of *M. thermoautotrophicum* ΔH on propionate oxidation by dilutions of a thermophilic enrichment culture[a]

Dilution	Without strain ΔH		With strain ΔH	
	Propionate degraded	Acetate formed	Propionate degraded	Acetate formed and then degraded
5×10^{-2}	5.8	5.8	19.7	19.7
25×10^{-4}	1.0	0.6	19.7	19.7
125×10^{-6}	0	0	19.7	19.7
625×10^{-8}	0	0	19.7	19.7

[a] Cultures were incubated for 6 weeks at 55°C. Controls showed that *M. thermoautotrophicum* ΔH was unable to degrade propionate and acetate. Values are millimoles per liter.

Defined cocultures with hydrogen- or formate-utilizing methanogens. During the enrichment experiments, spore-forming cells were observed. After an anaerobic heat treatment (30 min at 90°C or 10 min at 100°C), cultures had lost the ability to form methane with propionate, hydrogen, or formate as the substrate, indicating that the methanogens did not survive pasteurization. However, pasteurized cultures were able to oxidize propionate after inoculation in hydrogen-pregrown cultures of methanogens. By repeated pasteurization at 90°C for 30 min and growth in hydrogen-pregrown cultures of methanogens, we obtained propionate-oxidizing cultures with either *M. thermoautotrophicum* ΔH or *M. thermoformicicum* Z245 as the only methanogen. These cultures were used for further study. The ability to

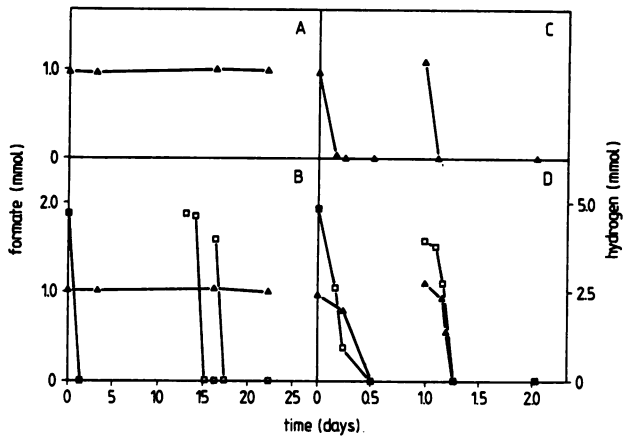

FIG. 3. Hydrogen (□) and formate (△) utilization by hydrogen-pregrown cultures of *M. thermoautotrophicum* ΔH and *M. thermoformicicum* Z245. Results for *M. thermoautotrophicum* ΔH incubated with formate (A) and hydrogen-formate (B) and for *M. thermoformicicum* Z245 incubated with formate (C) and hydrogen-formate (D) are shown. Data are expressed as millimoles per vial. Note the different time scales.

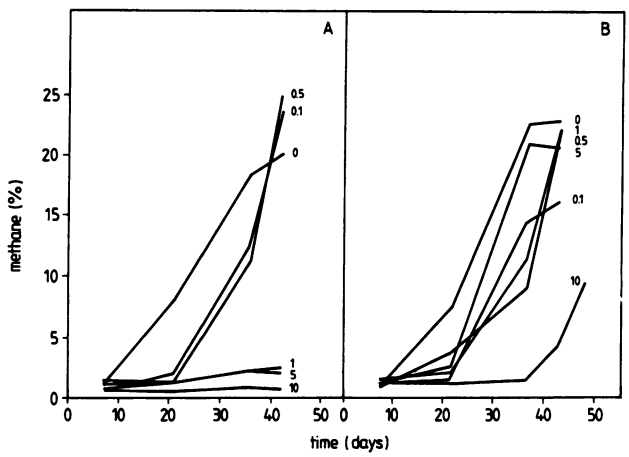

FIG. 4. Inhibition of methane formation from propionate by formate in the presence (A) and absence (B) of fumarate. The initial formate concentrations are indicated. The methanogen alone was unable to reduce fumarate with formate.

form spores was lost in both cocultures after repeated subcultivation; these cultures were susceptible to pasteurization. In some but not all cultures, spore formation was retrieved by addition of $MnCl_2$ (final concentration, 5 mM) and filter-sterilized effluent of the thermophilic upflow anaerobic sludge bed reactor from which the propionate-oxidizing bacteria had been enriched.

Role of formate and hydrogen. *M. thermoautotrophicum* ΔH was reported to use only hydrogen as an energy substrate (36). However, this methanogen was isolated in the absence of selenium and tungsten (36). These trace elements may be present in the formate dehydrogenases of methanogens (18, 19), and therefore, we reexamined formate utilization of *M. thermoautotrophicum* ΔH in the presence and absence of hydrogen in media with 0.1 μM selenium and tungsten. Figure 3A and B shows clearly that *M. thermoautotrophicum* ΔH does not use formate, either in the absence or in the presence of hydrogen. This finding proves that hydrogen transfer is the mechanism by which electrons are transferred from the propionate-oxidizing bacteria to this methanogen. In growing cultures with *M. thermoautotrophicum* ΔH, the hydrogen partial pressure was between 26 and 28 Pa (2.6×10^{-4} and 2.8×10^{-4} atm). In cultures in which the methanogen was inhibited by bromoethane sulfonate (10 mM), hydrogen partial pressures were only slightly higher (30 to 34 Pa). The threshold values of methanogens for hydrogen were dependent on the methane partial pressure. After growth of *M. thermoautotrophicum* ΔH with 80% H_2 and 20% CO_2 at a pressure of 172 kPa (1.7 atm), the hydrogen partial pressure was 10 to 15 Pa, whereas after growth with 8% H_2, 72% N_2, and 20% CO_2, this value was slightly lower (6 to 11 Pa). Comparable values were found in cultures with *M. thermoformicicum* Z245 (results not shown). In contrast with the *M. thermoautotrophicum* strain, this methanogen showed rapid growth with both formate and hydrogen-carbon dioxide as substrates (Fig. 3C and D). If product formation and growth remain coupled, a logarithmic increase in concentration can be used to estimate specific growth rates of propionate-oxidizing bacteria. On the basis of acetate production, we determined specific growth rates of propionate-oxidizing bacteria in culture with the two methanogens under different conditions. In stationary cultures with FeS, comparable growth rates were observed with *M. thermoformicicum* and *M. thermoautotrophicum* (0.15 and 0.14 day^{-1}, respectively). However, in shaken cultures, growth rates with the formate-utilizing strain were higher (0.19 versus 0.14 day^{-1} in media with FeS and 0.17 versus 0.04 day^{-1} in media without FeS). Remarkably, addition of formate to propionate-oxidizing cultures with strain ΔH led to a strong inhibition of propionate oxidation (Fig. 4A). At a formate concentration of 1 mM, propionate oxidation was completely suppressed. A measurable increase of the hydrogen partial pressure in propionate-oxidizing cultures due to the addition of formate was not observed, and formate was not degraded. Inhibition by formate could be neutralized by addition of the formate-utilizing methanogen (results not shown) or by addition of fumarate (Fig. 4B). Chemical analysis of cultures to which formate and fumarate were added showed that succinate was formed by a stoichiometric reduction of fumarate with formate. Growth rates and threshold values for hydrogen of *M. thermoautotrophicum* ΔH were not affected by 20 mM formate. In addition, this methanogenic strain was unable to reduce fumarate with formate.

Effect of aceticlastic methanogens. Besides low hydrogen concentrations, low acetate concentrations may be advantageous for propionate oxidation as well. With our media, we were not successful in growing *Methanothrix* sp. strain CALS-1, an organism which uses only acetate for growth (25). When enrichments were made at 55°C in acetate-containing media with thermophilic granules as a starting material, bacteria which resembled morphologically and physiologically the thermophilic acetate-utilizing methanogen described by Ahring and Westermann (1) came to the fore. A highly purified culture of rod-shaped bacteria with typical flat to blunted ends was obtained by repeated dilution in acetate-containing media. Besides acetate, the enrichment grew with formate and hydrogen-carbon dioxide as substrates, but it did not degrade propionate either in the absence or the presence of *M. thermoautotrophicum* ΔH. The effect of this aceticlastic methanogenic culture on propionate oxidation was investigated (Fig. 5). The fastest

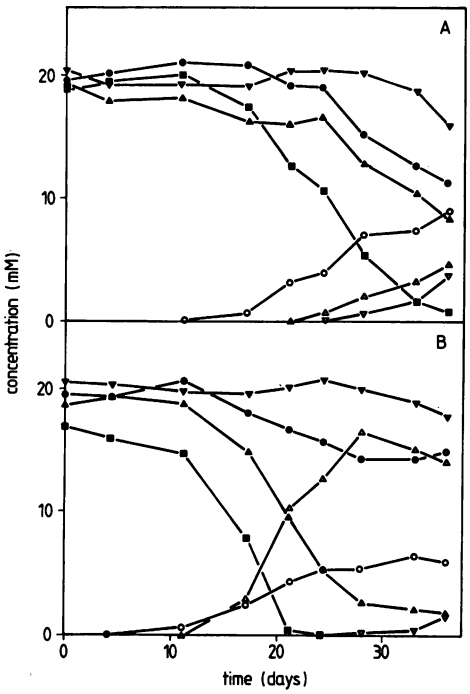

FIG. 5. Propionate oxidation (closed symbols) and acetate formation (open symbols) by stationary (A) and shaken (B) cultures of propionate-oxidizing bacteria without the addition of methanogens (▼ and ▽) and with addition of *M. thermoautotrophicum* ΔH (● and ○), aceticlastic methanogens (▲ and △), and *M. thermoautotrophicum* ΔH and aceticlastic methanogens (■). The methanogenic cultures were inoculated with a 50% inoculum, whereas the propionate-oxidizing culture was inoculated with a 2% inoculum. Note that acetate did not accumulate when both types of methanogens were present. It was ensured that in the absence of the propionate-oxidizing culture, propionate was not degraded.

propionate oxidation was observed in shaken cultures in which both types of methanogens were present. In that case, a specific growth rate of about 0.32 day^{-1} was calculated. Remarkably, when the aceticlastic methanogens were used in combination with hydrogenotrophic methanogens, acetate did not accumulate, whereas acetate accumulated when the aceticlastic methanogens were used alone. This indicates that the aceticlastic methanogens (i) prefer hydrogen to acetate as a substrate and (ii) have a lower affinity for hydrogen than *M. thermoautotrophicum* ΔH has.

Alternative electron acceptors. The propionate-oxidizing bacteria were unable to use inorganic or organic electron acceptors for growth on propionate. Sulfate, thiosulfate, sulfur, nitrate, nitrite, fumarate, or glycine could not replace methanogens for the removal of reducing equivalents. However, in the presence of *M. thermoautotrophicum* ΔH, fumarate was used as an additional electron acceptor in propionate oxidation. Addition of fumarate led to an enhancement of propionate oxidation, while fumarate was reduced to succinate (Table 3). It was confirmed that the methanogen alone was unable to reduce fumarate with formate. Growth in the presence of fumarate led to an increased cell yield of presumable propionate oxidizers; in media with 20 mM propionate, $(0.95 \pm 0.26) \times 10^8$ and $(1.75 \pm 0.23) \times 10^8$ cells per ml were counted in the absence and presence of fumarate, respectively. Propionate oxidizers and methanogens differed considerably in size and shape. The methanogen had a width two times smaller than that of the propionate oxidizer and formed irregular long rods.

TABLE 3. Effect of fumarate on propionate oxidation by the thermophilic enrichment culture[a]

Addition	Propionate degraded	Acetate formed	Fumarate degraded[b]	Succinate formed	Methane formed
None		1.1			0.9
Fumarate		1.1	0.4	1.0	0.5
Propionate	3.1	4.7			3.8
Fumarate + propionate	10.8	13.6	6.8	8.4	8.2

[a] Propionate-oxidizing culture was inoculated (5%) in hydrogen-pregrown cultures of *M. thermoautotrophicum* ΔH and incubated for 4 weeks at 55°C. Substrates were supplied at a concentration of 20 mM. Values are millimoles per liter.

[b] Fumarate was partly converted to malate; this is not taken into account here.

Optimum temperature. The optimum temperatures of *M. thermoautotrophicum* ΔH and *M. thermoformicicum* Z245 are 65 and 55°C, respectively (36, 37). The propionate oxidizers which were enriched at 55°C had an optimum temperature of 55°C, irrespective of the type of methanogen. Above 60 and below 45°C, no growth occurred in cultures with strain ΔH. No propionate oxidation occurred when bacteria were cultured with *Methanospirillum hungatii* DSM 864 at 37°C, showing that the enriched bacteria were clearly different from previously described mesophilic propionate oxidizers (7, 9, 21).

DISCUSSION

Results obtained in this study show clearly that the stability of methanogens against starvation is the determining factor for a successful enrichment of thermophilic, syntrophic, propionate-oxidizing bacteria. We measured maximum specific decay rates for *M. thermoautotrophicum* cells which were in suspended cultures and embedded in precipitates of FeS of 0.48 and 0.29 day^{-1}, respectively. Maximum specific growth rates of propionate oxidizers were strongly dependent on the type of methanogen and incubation conditions. For enrichment cultures and cultures in which hydrogenotrophic methanogens and aceticlastic methanogens were present, we measured specific growth rates between 0.25 and 0.32 day^{-1}, whereas in cultures with solely hydrogenotrophic methanogens, specific growth rates between 0.07 and 0.19 day^{-1} were determined. In all cases, the maximum specific growth rate was lower than the maximal decay rate of the methanogens. The decay rate of methanogens when cultures were supplied with limiting amounts of molecular hydrogen, as occurs during cultivation with propionate-oxidizing bacteria, is not known, but when small inoculum sizes were used (for example, the shaken culture with strain ΔH [Fig. 5]), hydrogen supply likely was not fast enough to prevent starvation. In the natural ecosystem, the effect of starvation may be of less importance because bacteria are immobilized and because methanogens receive substrate from other sources. In addition, methanogens which are more stable under starvation conditions may exist. On the basis of methane production, Wiegant et al. (34) calculated maximum specific growth rates for propionate oxidizers within intact methanogenic granules of about 0.72 day^{-1}. If this value is correct, it would indicate that the conditions for propionate oxidizers in our artificial systems are still suboptimal. Recently, we have shown that propio-

nate-grown mesophilic granules consist of microcolonies in which propionate-oxidizing bacteria and hydrogenotrophic methanogens are oriented close to each other. Disruption of the spatial orientation of bacteria in the granules resulted in a strong decrease in the rate of propionate oxidation (12).

The phenomenon that methanogens are the most sensitive organisms in syntrophic fatty acid degradation had not yet been considered in earlier reports. Most probably, growth rates of acetogens versus decay rates of methanogens in mesophilic cultures are less unfavorable. In thermophilic butyrate-oxidizing cocultures, starvation of methanogens seems to be of little importance as well. Thermophilic butyrate-oxidizing bacteria showed colony formation in a lawn of *M. thermoautotrophicum* within 4 weeks of incubation (14), and their specific growth rates were higher than those of the propionate oxidizers. Specific growth rates of 0.29 to 0.50 day^{-1} and 0.37 to 0.79 day^{-1} were measured in butyrate-degrading cocultures and tricultures, respectively (2).

Recently, it was proposed that formate transfer is an alternative mechanism by which electrons are transferred from acetogens to methanogens (8, 32). From our results, we cannot exclude the possibility that formate transfer does not play a role when propionate-oxidizing bacteria are grown in the presence of *M. thermoformicicum*. However, our results show clearly that formate transfer is not essential in our propionate-oxidizing cultures, because *M. thermoautotrophicum* ΔH is unable to metabolize formate. However, faster growth in cultures with *M. thermoformicicum* was observed. In view of the great variation in specific growth rates with each of the methanogens and because *M. thermoformicicum* is more stable under starvation conditions, it seems rather unlikely that formate is essential in cultures with *M. thermoformicicum*. In addition, the observation that the aceticlastic methanogens used acetate in the presence of *M. thermoautotrophicum* but not when applied alone cannot easily be explained if formate transfer is of any importance.

Although formate transfer does not play a role in propionate-oxidizing cultures with *M. thermoautotrophicum* ΔH and although this methanogen is not affected by formate, a strong inhibition of propionate oxidation in the presence of formate was found. This suggests that the metabolism of propionate oxidizers is directly affected by formate. The inhibition was abolished by addition of either a formate-utilizing methanogen or by addition of fumarate. The latter needs some further explanation. Research with mesophilic cocultures has shown that propionate oxidation occurs via the so-called succinate pathway (15–17, 21, 27). In this route, conversion of succinate to fumarate coupled with hydrogen formation is the most difficult step in the metabolism of propionate-oxidizing bacteria. In the presence of formate, propionate-oxidizing bacteria apparently are able to reduce fumarate with formate to succinate; the $\Delta G'$ of this reaction at 55°C is −79.5 kJ. Most likely, the presence of formate during propionate oxidation prevents the organisms from oxidizing succinate to fumarate.

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