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Hydrogen Partial Pressures in a Thermophilic Acetate-Oxidizing Methanogenic Coculture

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Hydrogen partial pressures were measured in a thermophilic coculture comprised of a eubacterial rod which oxidized acetate to H_2 and CO_2 and a hydrogenotrophic methanogen, *Methanobacterium* sp. strain THF. Zinder and Koch (S. H. Zinder and M. Koch, Arch. Microbiol. 138:263–272, 1984) originally predicted, on the basis of calculations of Gibbs free energies of reactions, that the H_2 partial pressure near the midpoint of growth of the coculture should be near 4 Pa (ca. 4×10^{-5} atm; ca. 0.024 μM dissolved H_2) for both organisms to be able to conserve energy for growth. H_2 partial pressures in the coculture were measured to be between 20 and 50 Pa (0.12 to 0.30 μM) during acetate utilization, approximately one order of magnitude higher than originally predicted. However, when ΔG_f (free energy of formation) values were corrected for 60°C by using the relationship $\Delta G_f = \Delta H_f - T\Delta S$ (ΔH_f is the enthalpy or heat of formation, ΔS is the entropy value, and T is the temperature in kelvins), the predicted value was near 15 Pa, in closer agreement with the experimentally determined values. The coculture also oxidized ethanol to acetate, a more thermodynamically favorable reaction than oxidation of acetate to CO_2. During ethanol oxidation, the H_2 partial pressure reached values as high as 200 Pa. Acetate was not used until after the ethanol was consumed and the H_2 partial pressure decreased to 40 to 50 Pa. After acetate utilization, H_2 partial pressures fell to approximately 10 Pa and remained there, indicating a threshold for H_2 utilization by the methanogen. Axenic cultures of the acetate-oxidizing organism were combined with pure cultures of either *Methanobacterium* sp. strain THF or *Methanobacterium thermoautotrophicum* ΔH to form reconstituted acetate-oxidizing cocultures. The H_2 partial pressures measured in both of these reconstituted cocultures were similar to those measured in the original acetate-oxidizing rod coculture. Since *M. thermoautotrophicum* ΔH did not use formate as a substrate, formate is not necessarily involved in interspecies electron transfer in this coculture.

Although the overall anaerobic degradation of organic matter to CH_4 and CO_2 is exergonic, certain steps in this process are sensitive to the buildup of the partial pressure of H_2. The anaerobic oxidation of alkanoic acids, such as propionate or butyrate, to acetate and H_2 is endergonic under standard conditions; however, such oxidation has been shown to occur when the organism carrying it out is in a syntrophic culture with a hydrogenotroph, such as a methanogen or a sulfate reducer (3, 13, 14, 18, 19). The hydrogenotroph maintains the H_2 partial pressure low enough that the fatty acid oxidation is exergonic (14, 21). It is clear that large additions of hydrogen lead to the blockage of syntrophic fatty acid oxidation in sewage sludge (7) or in syntrophic cultures (3, 15), but few measurements have been made of the actual H_2 levels allowing the reactions to proceed. The oxidation of fatty acids is considered to be essential for the proper functioning of anaerobic digestors (12, 14).

Conrad et al. (4, 6) measured the concentration of H_2 and various other metabolites in natural habitats, including lake sediments, sewage sludge, and the wetwood of cottonwood trees. They concluded that the oxidation of propionate and butyrate in Lake Mendota sediments and sewage sludge would be endergonic, despite the fact that the reactions apparently were occurring (17). They hypothesized a physical association among hydrogen producers and consumers (juxtaposition) that would form microenvironments in which the H_2 partial pressures would be lower than that measured in the bulk liquid (5). Compared to natural habitats, defined cocultures can provide clearer results concerning the effects of H_2 partial pressures on syntrophic reactions, since they usually only consist of two organisms carrying out a single set of reactions rather than a complex mixture of microorganisms carrying out several different reactions. Also, the potential physical association between the two partners can be directly observed, and slow-growing cocultures can be in equilibrium with the headspace (5, 16).

We have been studying the interactions in a two-membered thermophilic (60°C) methanogenic coculture which converted acetate to methane via interspecies hydrogen transfer (25). The coculture consisted of an acetate-oxidizing rod (AOR) which has been recently isolated and described (8) and a hydrogenotrophic methanogen, *Methanobacterium* sp. strain THF. Similar to the situation for the oxidation of higher alkanoic acids to acetate, the oxidation of acetate to CO_2 is endergonic under standard conditions (standard free energy $[\Delta G^{0\prime}] = +104.6$ kJ per reaction) and proceeds only because the H_2 partial pressure is kept low by the methanogen. Zinder and Koch (25) calculated that near the midpoint of growth of the culture, the H_2 partial pressure should be near 4 Pa (ca. 4×10^{-5} atm, ca. 0.024 μM dissolved H_2) for both organisms to obtain energy for growth. Here we present measurements of H_2 partial pressures in the headspace of cocultures oxidizing acetate and compare those measurements with the level predicted by thermodynamic equations.

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We also test whether formate, rather than H_2, is transferred between the two syntrophic partners.

MATERIALS AND METHODS

Media and culture conditions. The culture medium consisted of the following, in grams per liter: NH_4Cl, 0.5; K_2HPO_4, 0.4; $MgCl_2 \cdot 6H_2O$, 0.1; yeast extract, 0.1; and resazurin, 0.001. The medium also contained trace metals solution at 10 ml/liter (25). The medium was boiled under N_2, which was passed over hot copper coils to remove O_2, and was then reduced with 0.5 g of neutralized cysteine hydrochloride per liter. The medium was dispensed inside an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.) into glass serum vials or tubes which were sealed with butyl rubber stoppers (Bellco Glass, Inc., Vineland, N.J.). After the medium was autoclaved, the headspace was replaced with sterile O_2-scrubbed N_2 (70%)-CO_2 (30%) (Matheson Gas Co., Secaucus, N.J.), and the following sterile anaerobic solutions were added (final concentrations): $NaHCO_3$, 12 mM; $Na_2S \cdot 9H_2O$, 0.1 g/liter; and $CaCl_2 \cdot 2H_2O$, 0.1 g/liters. Carbon sources were added to the following final concentrations when appropriate: sodium acetate, 40 mM; and ethanol, 20 mM. Cultures grown on H_2 (80%)-CO_2 (20%) (Matheson) were grown in 200 ml of medium in 1-liter bottles (Bellco) in a 60°C shaking water bath. Other cultures were grown at 60°C under static conditions unless otherwise noted. "Reconstituted" cocultures were formed by inoculating H_2-CO_2-grown cultures of the AOR axenic culture (2% inoculum) into medium with ethanol, acetate, and an H_2-CO_2-grown culture of either *Methanobacterium* sp. strain THF of *Methanobacterium thermoautotrophicum* ΔH. Glass syringes were used for all manipulations to minimize exposure to oxygen.

Analytical methods. Methane was determined by using a model 550 gas chromatograph with a thermal conductivity detector (Gow-Mac Instrument Co., Bound Brook, N.J.) with He as a carrier gas. Hydrogen was determined by using an AGC series 100 gas chromatograph with a thermistor thermal conductivity detector (Hach Carle Chromatography Co., Loveland, Colo..). The column was packed with 80% Poropak N–20% Poropak Q (Supelco, Inc., Bellefonte, Pa.), the carrier gas was N_2, and the oven temperature was 25°C, as in the procedure described by Robinson and Tiedje (16). Gas samples were removed by using 1-ml Glaspak syringes (Fisher Scientific Co., Pittsburgh, Pa.) fitted with Mininert valves (Supelco) to maintain pressure. Partial pressures of H_2 were converted to molar concentrations by using a Henry Law constant of 0.6 mM H_2 per atm (101 kPa) at 60°C (Matheson Scientific Co. Unabridged Gas Data Book, vol. 2, 1974). The detection limit for H_2 was near 1 Pa. Standards for calibration of the gas chromatograph were 10-fold serial dilutions of H_2 mixed with N_2 and ranging from 10^0 to 10^5 Pa of H_2. The response of the detector was found to be linear for H_2 partial pressures up to 10^4 Pa.

Aqueous fermentation products were determined by high-performance liquid chromatography. An Altex 110A high-performance liquid chromatograph pump and a Knauer 71 refractive index detector (Rainin Instruments, Inc., Woburn, Mass.) fitted with an HPX-87H organic acid analysis column (Bio-Rad Laboratories, Richmond, Calif.) were used for the quantification of acetate and ethanol. The column was operated at room temperature, and the solvent was 6.5 mM sulfuric acid, as previously described by Zinder and Koch (25).

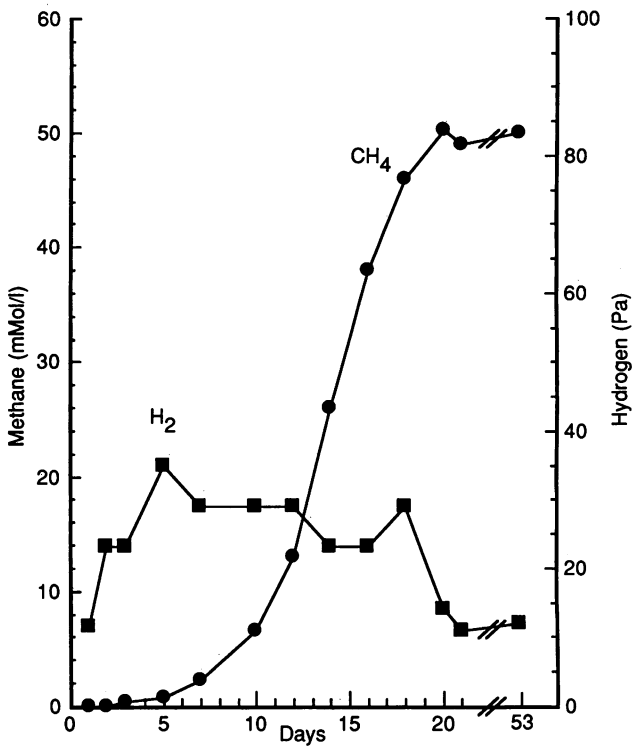

FIG. 1. H_2 partial pressures and methane production (expressed as millimoles produced per liter of culture medium) in the AOR coculture during growth on 40 mM acetate.

RESULTS

H_2 partial pressures in acetate-grown cocultures. Measurements of H_2in the headspaces of AOR-*Methanobacterium* sp. strain THF cocultures growing on acetate showed that the H_2 partial pressure was between 20 and 40 Pa (0.12 to 0.24 μM) during active acetate oxidation (Fig. 1). The H_2 partial pressure decreased to 12 to 14 Pa after acetate oxidation was complete and remained at that level, even after several weeks of incubation. It was important to assure that there was adequate gas transfer between the gas and liquid phases so that the H_2 partial pressures that we measured were representative of dissolved H_2 in the liquid phase (5, 16). To greatly increase gas transfer we incubated cultures in a 60°C shaking water bath at 150 rpm. H_2 levels in such cultures were also 20 to 40 Pa (data not shown), indicating that gas transfer to the headspace was adequate during incubation in static cultures.

H_2 levels in ethanol-grown cocultures. We have also found that the AOR can grow in a syntrophic coculture but not axenically on ethanol (8). When grown on ethanol and acetate, the coculture used ethanol first, oxidizing it to acetate (Fig. 2). The H_2 partial pressure was much higher (up to 200 Pa) during growth on ethanol than during growth on acetate. Acetate utilization did not start until the H_2 partial pressure fell to below 50 Pa. After the acetate was consumed, the H_2 partial pressure again fell to 12 Pa and remained there.

Is interspecies formate transfer necessarily involved? Reconstitution of the AOR with *M. thermoautotrophicum* ΔH. Since *Methanobacterium* sp. strain THF is capable of using formate (25), it was possible that some or all of the interspecies electron transfer observed was due to formate rather

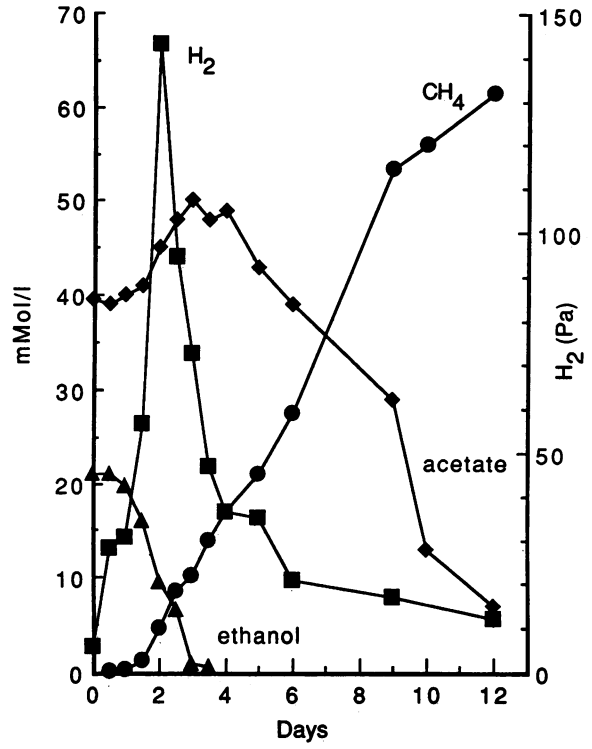

FIG. 2. Methane production, H_2 partial pressures, and ethanol and acetate concentrations in the AOR coculture during growth on 20 mM ethanol and 40 mM acetate. mMol, Millimoles.

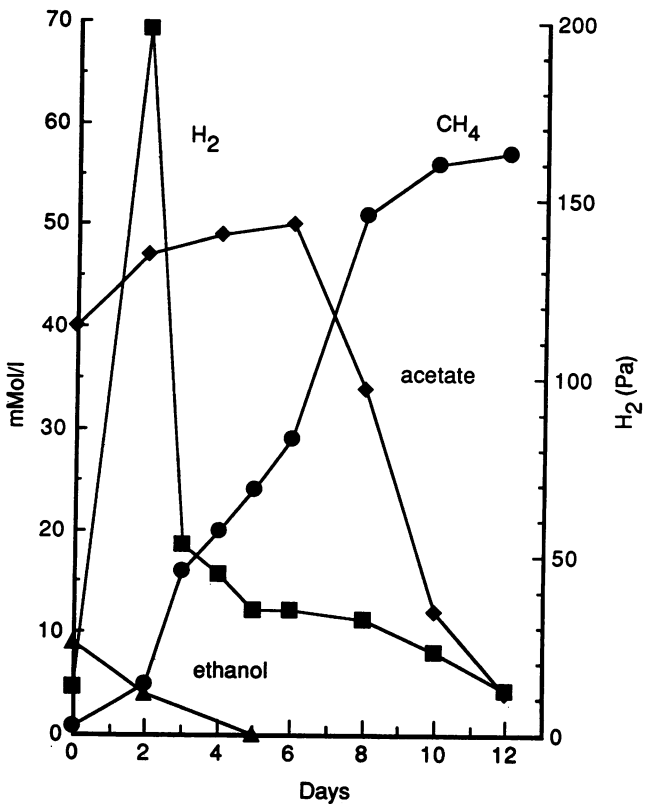

FIG. 3. Methane production, H_2 partial pressures, and ethanol and acetate concentrations in an acetate-oxidizing biculture reconstituted from axenic cultures of the AOR and *M. thermoautotrophicum* ΔH during growth on 10 mM ethanol and 40 mM acetate. mMol, Millimoles.

than H_2 transfer. To test this possibility, we used the axenic AOR culture grown on H_2-CO_2 to reconstitute the coculture on ethanol-acetate with either *Methanobacterium* sp. strain THF or *M. thermoautotrophicum* ΔH, which does not use formate (24). We reconfirmed the inability of strain ΔH to use 50 mM sodium formate by incubating cultures for up to 2 months at 60°C with formate. We also incubated cultures of strain ΔH with formate and H_2-CO_2 together. Only H_2 was used, while strain THF used both substrates (data not shown). Thus, if formate was obligatorily involved in interspecies electron transport, growth of the AOR coculture with strain ΔH should be greatly impaired when compared with growth of the AOR coculture with strain THF.

Both reconstituted cocultures grew and switched from ethanol to acetate. Figure 3 shows the results for *M. thermoautotrophicum* ΔH. The H_2 level at which acetate utilization started was near 60 Pa, very similar to the value observed in the original coculture. Growth of the coculture with *M. thermoautotrophicum* ΔH was essentially indistinguishable from that of either the original coculture or a reconstituted coculture with *Methanobacterium* sp. strain THF. Fermentation product analysis of culture supernatants showed that the accumulation of formate was below the detection limit of 10 μM in cocultures with either *Methanobacterium* sp. strain THF or *M. thermoautotrophicum* ΔH as the methanogenic partner.

DISCUSSION

The measured levels of H_2 in the syntrophic acetate-oxidizing coculture during growth were nearly one order of magnitude higher than that originally predicted by Zinder and Koch (25). The basis of those calculations was to use $\Delta G^{0\prime}$ values of +104.6 kJ and −135.6 kJ per reaction, respectively (22), for the following reactions:

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+ \quad (1)$$

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

The AOR carries out the reaction in equation 1, while the methanogen carries out the reaction in equation 2 (25). The Gibbs free energy ($\Delta G'$) value for the reaction at the incubation temperature of 60°C (333 K) under conditions approximating the midpoint of growth of the culture on 40 mM acetate, at which the acetate and HCO_3^- concentrations would be near 20 and 30 mM, respectively, and the CH_4 partial pressure would be near 35 kPa, was calculated by using the free-energy form of the Nernst equation (2, 21):

$$\Delta G' = \Delta G^{0\prime} + RT \ln \frac{(HCO_3^-)^2 (H_2)^4}{CH_3COO^-}$$

$$= 104.6 + 6.36 \log \frac{(0.03)^2}{(0.02)} + 6.36 \log (H_2)^4 \quad (3)$$

where R is the ideal gas constant, T is the temperature in kelvins, and the values in parentheses represent either the molar concentrations of solutes or the partial pressures of the gases in atmospheres. The predicted maximum value for hydrogen of 17 Pa (1.7×10^{-4} atm) was obtained by solving this equation for H_2 when $\Delta G' = 0$, which would be the point

at which the AOR could conserve no energy from the reaction in equation 1. The minimum value of 0.9 Pa was calculated in a similar fashion for the methanogenic reaction (equation 2), and a value of 4 Pa, the mean of the logarithms of the two values, would be the predicted H_2 partial pressure if both organisms conserved equal energy from their reactions.

One potential explanation for the discrepancy between the predicted and measured values of H_2 was that the reactions were being carried out at 60°C, a temperature considerably higher than the standard temperature of 25°C. The standard free energy of formation (ΔG_f) values for the molecules at 25°C can be corrected to 60°C by taking the entropy of formation into account by using the following equation (2, 20):

$$\Delta G_f = \Delta H_f - T\Delta S \quad (4)$$

The enthalpy values (ΔH_f) and entropy values (ΔS) were obtained from a standard reference (2) and can be considered to be essentially constant over the temperature range of 0 to 80°C (2). Use of the corrected values of ΔG_f yielded a $\Delta G^{0\prime}$ of +88 kJ per reaction at 60°C rather than +104.6 kJ per reaction at 25°C. Solving equation 3 for $\Delta G' = 0$ with the corrected values yielded a maximum value for H_2 of 74 Pa. Following similar procedures, a minimum value for H_2 of 2.6 Pa in equation 2 was arrived at, and if both organisms conserved equal energy the value for H_2 partial pressure would near 14 Pa. Thus, the obtained values of 20 to 40 Pa were within this range. Another correction to consider is that chemical activities rather than concentrations of solutes should be used in the calculation of free energies. It was found, using the extended Debye-Huckel equation (2, 20), that such a correction had a negligible effect on the free energies calculated.

Figure 4 represents the predicted effects of temperature on the upper ($\Delta G' = 0$ in equation 1) and lower ($\Delta G' = 0$ in equation 2) boundaries for H_2 partial pressures in syntrophic cocultures oxidizing acetate at temperatures of 0 to 80°C. Temperature effects were due to the T value in both equations 3 and 4. These effects were highly significant, with both boundaries increasing over two orders of magnitude as the temperature increased from 0 to 80°C. The prediction that H_2 partial pressures should be lower in mesophilic cocultures with acetate than in thermophilic ones cannot be tested at this time, since a mesophilic acetate-oxidizing coculture has not presently been described. Also shown in Figure 4 are the calculated upper boundaries at 25 and 60°C for butyrate oxidation to acetate and H_2, the reaction carried out by *Syntrophomonas wolfei* (15). The value at 25°C was in agreement with the one calculated by McInerney and Bryant (14) when differences in concentrations of products and reactants were taken into account.

The measured value of >0.1 Pa for a mesophilic (35°C) butyrate-oxidizing enrichment culture reported by Tomei et al. (22) is clearly below the lower limit for methanogenesis (0.5 Pa), according to Fig. 4, as are the values reported by Conrad et al. (4–6) for lake sediments. It is possible that sulfate-reducing bacteria may be depressing the H_2 partial pressures in these systems. The H_2 partial pressure range of 10 to 100 Pa reported for a 60°C coculture by Ahring and Westermann (1) is within the limits predicted. If the relationship of H_2 partial pressures to temperature presented in Fig. 4 is valid, it implies that H_2 partial pressures should be considerably higher in high-temperature anaerobic ecosystems than in corresponding lower-temperature ones.

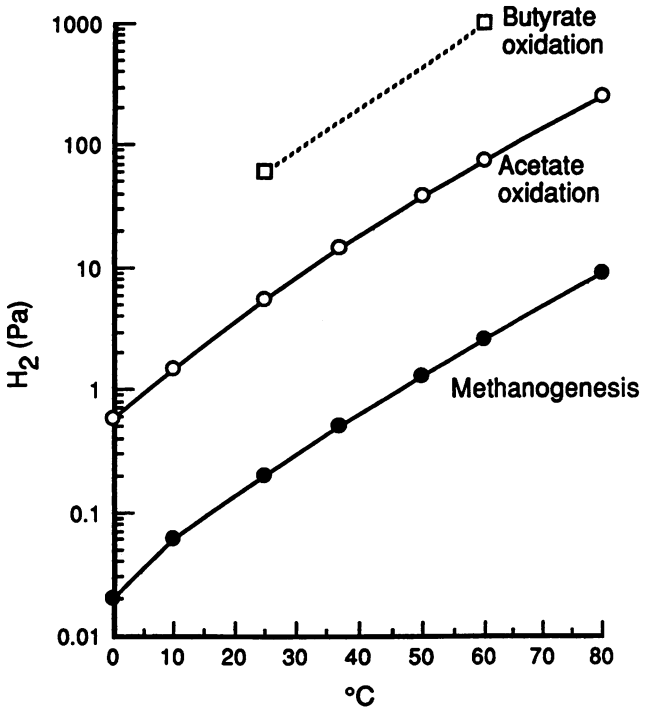

FIG. 4. Effect of temperature on the H_2 partial pressures at which $\Delta G' = 0$ for various reactions under conditions similar to those at the midpoint of growth of a syntrophic coculture. The reactions are acetate oxidation ($CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$), methanogenesis ($4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$), and butyrate oxidation ($CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2\,CH_3COO^- + 2H_2 + H^+$). For acetate oxidation and methanogenesis, the concentrations of the products and reactants are as follows: acetate, 20 mM; HCO_3^-, 30 mM; and CH_4, 0.35 atm. For butyrate oxidation, the concentrations of products and reactants are as follows: butyrate, 10 mM; and acetate, 10 mM.

The levels of H_2 seen in the acetate-oxidizing coculture are similar to those reported by Lovley and Ferry (10) for *Methanosarcina thermophila* TM-1 growing at 50°C on acetate. During methanogenesis from acetate, *M. thermophila* maintained H_2 near a partial pressure of 50 Pa, either producing H_2 to that level when the partial pressure was decreased or consuming H_2 to that level when it was increased. This H_2 may have come from hydrogen cycling during acetate splitting or from acetate oxidation (26). The mesophile *Methanosarcina acetivorans* equilibrated H_2 to a partial pressure near 20 Pa, a value considerably lower and consistent with the effect predicted by Fig. 4.

The coculture oxidized ethanol to acetate during the first phase of growth on an ethanol-acetate mixture, in contrast to the results with *Desulfotomaculum acetoxidans*, which completely oxidized ethanol to CO_2 (23). H_2 partial pressures were considerably higher (150 to 200 Pa) during growth on ethanol, as expected, since the $\Delta G^{0\prime}$ for ethanol oxidation is +9.6 kJ per reaction, indicating that ethanol oxidation is considerably more favorable than acetate oxidation (21). That acetate was not consumed until H_2 partial pressures were below 50 Pa is consistent with the thermodynamic arguments previously presented.

When methanogenesis ceased, H_2 partial pressures in the acetate-oxidizing coculture decreased to near 12 to 14 Pa and were not reduced below that level even after prolonged incubations. This apparent threshold level is higher than the

threshold of 6.5 to 9.5 Pa observed for mesophilic (37°C) H_2-utilizing methanogens in pure cultures (9) and the threshold of ca. 1 Pa for methanogenic lake sediments incubated at 20°C (10). At a partial pressure of 12 Pa, the free energy for the methanogenic reaction (equation 2) under the conditions in Fig. 4 would be near −17 kJ per reaction.

Methanobacterium sp. strain THF was shown to use formate as a growth substrate (25), and it is possible that interspecies electron transfer might have been mediated by formate in this coculture. *M. thermoautotrophicum* ΔH, which does not use formate (24), served as an adequate methanogenic partner, yielding results indistinguishable from those obtained with *Methanobacterium* sp. strain THF. No formate was detected in cocultures established with *M. thermoautotrophicum* ΔH or with *Methanobacterium* sp. strain THF. These results make it unlikely that formate was involved in interspecies electron transfer in this instance.

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