

Syntrophic Growth on Formate: a New Microbial Niche in Anoxic Environments[∇]

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Anaerobic syntrophic associations of fermentative bacteria and methanogenic archaea operate at the thermodynamic limits of life. The interspecies transfer of electrons from formate or hydrogen as a substrate for the methanogens is key. Contrary requirements of syntrophs and methanogens for growth-sustaining product and substrate concentrations keep the formate and hydrogen concentrations low and within a narrow range. Since formate is a direct substrate for methanogens, a niche for microorganisms that grow by the conversion of formate to hydrogen plus bicarbonate—or vice versa—may seem unlikely. Here we report experimental evidence for growth on formate by syntrophic communities of (i) *Moorella* sp. strain AMP in coculture with a thermophilic hydrogen-consuming *Methanothermobacter* species and of (ii) *Desulfovibrio* sp. strain G11 in coculture with a mesophilic hydrogen consumer, *Methanobrevibacter arboriphilus* AZ. In pure culture, neither *Moorella* sp. strain AMP, nor *Desulfovibrio* sp. strain G11, nor the methanogens grow on formate alone. These results imply the existence of a previously unrecognized microbial niche in anoxic environments.

Much attention is paid to the environmental conditions that limit microbial growth and activity (24, 30, 31), such as high salt concentrations, high pressure, high and low pHs, high and low temperatures, and combinations thereof (3, 11, 23, 30, 32, 42). Less attention has been given to the thermodynamic limits of microbial life, although these are the most fundamental limits for any life form (19). These limits are approached in methanogenic environments, where syntrophic associations of anaerobic bacteria and methanogenic archaea obtain energy for growth from catalyzing pathways that operate close to thermodynamic equilibrium (ΔG , ~ 0 kJ/mol) (20, 38). Methanogenic communities are generally schematized as four different functional groups (or guilds) of bacteria and archaea. Primary fermenters convert complex material into substrates for a group of secondary fermenters, also known as syntrophs. The syntrophs obligately depend on two groups of methanogens, one that uses hydrogen and formate and another that uses acetate (9, 38). For thermodynamic reasons, growth of the syntrophs is sustainable only through the removal of their waste products by the methanogens. Hydrogen is the main electron carrier in such syntrophic associations, but formate is important too, especially in associations where electron fluxes are high (5, 8, 41). It is assumed that formate and hydrogen are in thermodynamic equilibrium (26, 44) (Table 1), but this is not always the case. For instance, measurements in a shallow methanogenic aquifer in Denmark have indicated a potential energy gain of 5 to 10 kJ/mol electrons for the conversion of formate to H₂ and bicarbonate (14). This implies a previously unrecognized niche for organisms that are able to catalyze this reaction.

Hydrolytic cleavage of formate to H₂ and bicarbonate has been described before (2, 7, 12, 29), but it has never been shown before that this can be coupled to growth (Table 1). Formate hydrogen lyase has been proposed to be coupled to energy conservation (15). Guyot and Brauman have reported formate-based coupling between a sulfate reducer and a non-formate-using methanogen, but growth was not demonstrated (12). Here we describe experiments that show that bacteria are able to grow by the conversion of formate to H₂ and bicarbonate, provided that hydrogen is consumed by a methanogen.

MATERIALS AND METHODS

Strains and source of microorganisms. *Moorella* sp. strain AMP (DSMZ 21394; GenBank accession number of the 16S rRNA gene sequence, AY884087) and *Methanothermobacter* sp. strain NJ1 were isolated from a methanol-degrading enrichment culture from a thermophilic upflow anaerobic sludge blanket reactor as described by Paulo et al. (35) and maintained routinely on methanol and H₂-CO₂, respectively. *Desulfovibrio* sp. strain G11 (DSMZ 7057) and *Methanobrevibacter arboriphilus* AZ (DSMZ 744) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and maintained routinely on formate plus sulfate and H₂-CO₂, respectively.

Media and growth conditions. Media and growth conditions were as described previously (21, 35). All media used for the coculture studies were without addition of carbon and energy sources other than formate. For the thermophilic (65°C) coculture studies, pregrown pure cultures of *Moorella* sp. strain AMP on methanol and *Methanothermobacter* sp. strain NJ1 on H₂-CO₂ were inoculated (10%, inoculum size) into fresh mineral medium with 60 mM formate as the sole energy source. For the mesophilic (37°C) coculture studies, a pure culture of *Desulfovibrio* sp. strain G11 (10%, vol/vol) was inoculated into a dense culture of *M. arboriphilus* AZ that was pregrown on H₂-CO₂. Subcultures were made after 40 mM formate was consumed until stable cocultures were obtained. This repeated transfer was performed at least five times.

All inoculations and transfers were done aseptically with sterile needles and syringes, and all cultures were incubated statically in the dark.

Analytical and other methods. Growth and growth yields were determined by measuring the increase in optical density at 600 nm, cell number counting with a Burkert-Türk counting chamber, and dry weight content measurements by standard techniques.

Gas (H₂ and CH₄) samples were analyzed by gas chromatography with a Shimadzu GC-14B (Shimadzu, Kyoto, Japan) equipped with a packed column

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TABLE 1. Gibbs free-energy changes for the reactions involved in syntrophic conversion of formate^a

Reaction	$\Delta G^{0'}$ at 25°C	$\Delta G'^b$ at 65°C
$4\text{HCOO}^- + 4\text{H}_2\text{O} \rightarrow 4\text{H}_2 + 4\text{HCO}_3^-$	+5.2	-6.3
$4\text{H}_2 + \text{HCO}_3^- + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{H}_2\text{O}$	-135.6	-146.2
$4\text{HCOO}^- + \text{H}_2\text{O} + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{HCO}_3^-$	-130.4	-152.5

^a Data were obtained or calculated from reference 1.

^b Gibbs free energy (kJ reaction⁻¹) was calculated under standard conditions (solute concentration of 1 M, pH of 7, and partial pressure of gas of 10⁵ Pa).

(Molsieve 13 × 60/80 mesh, 2-m length, 2.4-mm internal diameter; Varian, Middelburg, The Netherlands) and a thermal conductivity detector. The oven temperature was 100°C, and the injector and detector temperatures were 90 and 150°C, respectively. Argon was the carrier gas at a flow rate of 30 ml min⁻¹. Formate was analyzed by high-pressure liquid chromatography from centrifuged (10,000 × g, 10 min) samples of the culture media. Formate was measured with a Polyspher OA HY column (300 by 6.5 mm; Merck, Darmstadt, Germany) and an RI SE-61 refractive index detector (Shodex, Tokyo, Japan). The mobile phase was 0.01 N H₂SO₄ at a flow rate of 0.6 ml min⁻¹. The column temperature was 60°C.

Thermodynamic calculations were done as described by Amend and Shock (1). Temperature corrections for 65°C were made by linear interpolation from tabulated values for 55 and 70°C. This method yielded essentially the same results as the use of the Gibbs-Helmholtz equation for temperature correction as described by Hanselmann (13). Corrections for the actual concentrations of substrates were made with the Nernst equation (27, 40). Comparative analyses of genome sequences were performed with the integrated microbial genomes system (28), which is available from the U.S. Department of Energy Joint Genome Institute (www.jgi.doe.gov).

RESULTS AND DISCUSSION

Two different defined communities were studied, a thermophilic community consisting of *Moorella* sp. strain AMP in coculture with *Methanothermobacter* sp. strain NJ1 and a mesophilic community consisting of *Desulfovibrio* sp. strain G11 in coculture with *M. arboriphilus* AZ. Both methanogens can only use H₂ as an electron donor.

Moorella sp. strain AMP and *Methanothermobacter* sp. strain NJ1 were isolated from a methanogenic bioreactor operated at 55°C (35). Based on 16S rRNA gene sequence analysis, strain AMP was closely related to *Moorella thermoacetica* and *Moorella thermoautotrophica*. The sequence was 98% identical to both *Moorella* strains. However, the new isolate had the special property of growth on CO, forming H₂ rather than acetate as the end product. Strain NJ1 was a hydrogen-utilizing methanogen; its 16S rRNA sequence was 99.5% identical to that of *Methanothermobacter thermoautotrophicus* ΔH (21).

Moorella sp. strain AMP grew on formate when thiosulfate was added as an electron acceptor but did not grow when thiosulfate was replaced with sulfate, nitrate, or fumarate. In the absence of thiosulfate, H₂ gradually accumulated to a partial pressure of 2,000 Pa. Removal of H₂ from the headspace via flushing resulted again in accumulation of H₂ (21). These observations indicate that accumulation of H₂ was inhibitory to the conversion of formate. Therefore, we tested whether *Methanothermobacter* sp. strain NJ1 could serve as an alternative electron acceptor. A coculture of *Moorella* sp. strain AMP and *Methanothermobacter* sp. strain NJ1 grew at 65°C on formate as the sole carbon and energy substrate (Fig. 1), while the individual pure cultures did not grow in formate-containing media (data not shown). Consumption of 2.71 ± 0.13 mmol formate

resulted in the formation of 0.60 ± 0.04 mmol CH₄, which is consistent with the reaction $4\text{HCOO}^- + \text{H}_2\text{O} + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{HCO}_3^-$, and gave rise to an increase in the total cell concentration from $9.0 \times 10^6 \pm 2.8 \times 10^6$ to $7.5 \times 10^7 \pm 1.4 \times 10^7$ /ml. In the coculture, hydrogen levels were between 10 and 150 Pa. Under these conditions, Gibbs free-energy changes ranged between -16 and -29 kJ/mol H₂ for the conversion of formate into H₂ and bicarbonate and between -9 and -12 kJ/mol H₂ for H₂-driven methanogenesis. Taken together, these data indicate that interspecies hydrogen transfer is essential to sustain the growth of the coculture and that *Moorella* sp. strain AMP can grow by the conversion of formate to H₂ and bicarbonate when the hydrogen concentration is kept low.

Moorella sp. strain AMP grew on carbon monoxide in pure culture. Carbon monoxide is converted into H₂ and bicarbonate in a fashion similar to that described for *Carboxydothemus hydrogenoformans* and several other gram-positive bacteria (17). The energetics of CO conversion ($\text{CO} + 2\text{H}_2\text{O} \rightarrow \text{H}_2 + \text{HCO}_3^- + \text{H}^+$; $\Delta G^{0'} = -15.4$ kJ at 25°C and $\Delta G' = -12.4$ kJ at 65°C) is more favorable than the energetics of formate conversion ($\text{HCOO}^- + \text{H}_2\text{O} \rightarrow \text{H}_2 + \text{HCO}_3^-$; $\Delta G^{0'} = 1.1$ kJ at 25°C and $\Delta G' = -1.6$ kJ at 65°C). Thus, CO oxidation allows growth even if the partial pressure of H₂ is high (17, 18). In *Carboxydothemus hydrogenoformans*, there is compelling

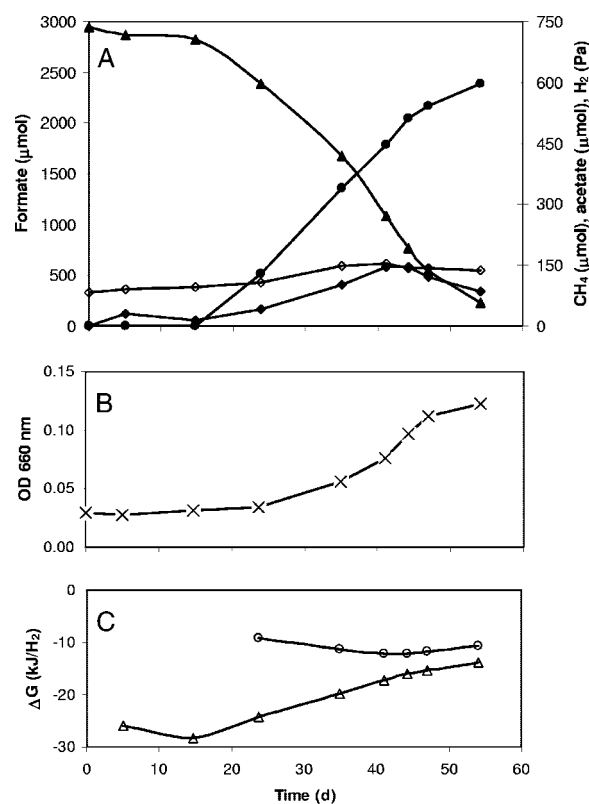


FIG. 1. Syntrophic growth on formate by a coculture of *Moorella* sp. strain AMP and *Methanothermobacter* sp. strain NJ1. (A) Changes in formate (▲), methane (●), hydrogen (◆), and acetate (◇). (B) Growth. OD 660 nm, optical density at 660 nm. (C) Actual Gibbs free-energy changes for formate degradation to H₂ and bicarbonate (Δ) and methane formation from H₂ and bicarbonate (○). Data are averages of duplicate incubations; the experiment was repeated once with essentially the same results.

TABLE 2. Genes and gene clusters possibly involved in formate conversion and hydrogen production by *M. thermoacetica* and *D. vulgaris*^a

Organism and enzyme	Localization ^b	Locus tag (GenBank accession no.)
<i>M. thermoacetica</i>		
Formate dehydrogenase	Outside, membrane integrated	Moth_0450-0452
Formate dehydrogenase	Cytoplasm	Moth_2312-2314 (U73807)
Formate hydrogen lyase	Cytoplasm, membrane integrated	Moth_2174-2193
Fe-only hydrogenase	Cytoplasm	Moth_1717-1719
<i>D. vulgaris</i>		
Formate dehydrogenase	Periplasm	DVU2481-2484
Formate dehydrogenase	Periplasm	DVU2809-2812
Formate dehydrogenase	Cytoplasm	DVU0587-0588
Hydrogenase (EchA, -B, -C, -D, -E, -F)	Cytoplasm, membrane integrated	DVU0429-0434
Hydrogenase (Ech-CO dehydrogenase)	Cytoplasm, membrane integrated	DVU2286-2293
Hydrogenase	Cytoplasm	DVU0325-0326
Hydrogenase, Fe only	Periplasm	DVU1769-1770
Hydrogenase NiFe isozyme 1	Periplasm	DVU1921-1922
Hydrogenase NiFeSe	Periplasm	DVU1917-1918
Hydrogenase NiFe isozyme 2	Periplasm	DVU2525-2526
Hydrogenase Mvr/hdr type	Cytoplasm	DVU2399-2404

^a www.jgi.doe.gov and NCBI GenBank.

^b Localization of the proteins was predicted by using SignalP and TMHMM online software (4, 33).

evidence that the organism conserves energy via a novel CO-oxidizing, H₂-evolving enzyme complex. The key for energy conservation is that the hydrogenase part of the proton-consuming-H₂-generating enzyme complex is located at the cytoplasmic site of the cell membrane (15). It is possible that *Moorella* sp. strain AMP conserves energy from the formate-hydrogen-lyase reaction via an analogous enzyme complex with an energy-conserving hydrogenase located at the cytoplasmic side of the cell membrane. Support for this hypothesis was obtained via an analysis of the genome of *Moorella thermoacetica*, the closest relative of *Moorella* sp. strain AMP, with 98% 16S RNA sequence identity. In *M. thermoacetica*, the genes that encode the constituents of a putative energy-conserving formate hydrogen lyase complex are all located in one operon. *M. thermoacetica* also possesses a membrane-integrated formate dehydrogenase (Table 2) that can oxidize formate at the outside of the membrane, thus generating a proton gradient over the membrane (Fig. 2) (36). The electrons produced are transferred across the membrane to a hydrogenase. Energy from the resulting proton gradient is harnessed via a membrane-integrated ATPase.

On the basis of these findings, it seems prudent to also test *M. thermoacetica* for the ability to grow syntrophically on formate. However, testing this experimentally is confounded by the fact that *M. thermoacetica* can grow on formate in pure culture via a different pathway whereby formate is converted to acetate ($4\text{HCOO}^- + \text{H}^+ \rightarrow \text{CH}_3\text{COO}^- + 2\text{HCO}_3^-$; $\Delta G^{0'} = -99.7$ kJ at 25°C and $\Delta G' = -95.2$ kJ at 65°C). Indeed *M. thermoacetica* is the model organism with which the pathway for the formation of acetate from hydrogen and formate was elucidated first (10). *Moorella* sp. strain AMP cannot grow homoacetogenically on H₂-CO₂ or formate, probably because it lacks cytochrome *b* (21).

To further explore the occurrence of the ability to grow by the conversion of formate to H₂ and bicarbonate, a mesophilic bacterium, *Desulfovibrio* sp. strain G11, was tested in coculture with a methanogen that could only use H₂ as an electron donor

(*M. arboriphilus* AZ). A coculture of *Desulfovibrio* sp. strain G11 and *M. arboriphilus* AZ grew well on formate as the sole carbon and energy substrate (Fig. 3), while the individual pure cultures did not grow in formate-containing media (data not shown). The growth yield of the coculture was 0.52 ± 0.12 g (dry weight)/mol of formate. Since floc formation was observed during syntrophic growth, growth could not be quantified by following the increase in turbidity. These flocs consisted of *Desulfovibrio* sp. strain G11 and the methanogen. The close proximity of the two microorganisms is beneficial for interspecies hydrogen transfer.

In the coculture, hydrogen levels were between 40 and 100 Pa (Fig. 3). Formate degradation resulted in a nearly stoichiometric formation of methane (1 mol of CH₄/4 mol of formate degraded) under transient accumulation of H₂ to a level of ~100 Pa. Under these conditions, Gibbs free-energy changes ranged between -17 and -19 kJ/mol H₂ for the conversion of formate into H₂ and bicarbonate and between -12 and -17 kJ/mol H₂ for H₂-driven methanogenesis. Formate was metabolized at a rate of ~100 μmol/day.

Desulfovibrio sp. strain G11 is not able to grow in pure culture by the conversion of formate to H₂ and bicarbonate. However, in pure culture it converts formate, resulting in a gradual accumulation of 100 Pa of hydrogen in the gas phase, after which the conversion of formate stopped. Removal of H₂ from the headspace via flushing resulted again in accumulation of H₂. A similar observation was done for *Desulfovibrio* sp. strain FOX1 (37). It is unclear if this bacterium is able to grow in pure culture from this conversion or growth was supported by the degradation of biomass, yeast extract, or other sources of organic carbon in the growth medium.

Desulfovibrio sp. strain G11 is a close relative of *Desulfovibrio vulgaris* strain Hildenborough, for which the genome sequence is available (16). We have also tested *D. vulgaris* for the ability to grow on formate in coculture with *M. arboriphilus*. *D. vulgaris* is indeed able to grow on formate with a syntrophic partner, but a stable consortium could only be obtained in the

thermodynamic oddity, but it has now been shown to be feasible and occur in various situations, e.g., in Lake Kinneret sediments (34), in subsurface petroleum reservoirs (22), and in other environments with long solid retention times (39). Therefore, this type of metabolism, while seemingly paradoxical in the context of the existence of acetoclastic and formate-utilizing methanogens, may actually be a more fundamental component of methanogenic organic-carbon-mineralizing systems than previously recognized. On the other hand, since the discovery by Bryant et al. (6) that *Methanobacillus omelianskii* is not a pure culture but a syntrophic coculture, ethanol is a known substrate for syntrophic communities. Moreover, some methanogens are known to use ethanol or isopropanol directly as an electron donor for methanogenesis (25, 43). Further research is needed to get insight into the environmental conditions under which substrates are degraded by methanogens alone or by syntrophic communities.

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