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Hydrogen or formate: Alternative key players in methanogenic degradation

Bernhard Schink, Dominik Montag, Anja Keller and Nicolai Müller*
Department of Biology, Microbial Ecology, University of Konstanz, Konstanz, D-78457, Germany

Summary

Hydrogen and formate are important electron carriers in methanogenic degradation in anoxic environments such as sediments, sewage sludge digestors and biogas reactors. Especially in the terminal steps of methanogenesis, they determine the energy budgets of secondary (syntrophically) fermenting bacteria and their methanogenic partners. The literature provides considerable data on hydrogen pool sizes in such habitats, but little data exist for formate concentrations due to technical difficulties in formate determination at low concentration. Recent evidence from biochemical and molecular biological studies indicates that several secondary fermenters can use both hydrogen and formate for electron release, and may do so even simultaneously. Numerous strictly anaerobic bacteria contain enzymes which equilibrate hydrogen and formate pools to energetically equal values, and recent measurements in sewage digestors and biogas reactors indicate that – beyond occasional fluctuations - the pool sizes of hydrogen and formate are indeed energetically nearly equivalent. Nonetheless, a thermophilic archaeon from a submarine hydrothermal vent, *Thermococcus onnurineus*, can obtain ATP from the conversion of formate to hydrogen plus bicarbonate at 80°C, indicating that at least in this extreme environment the pools of formate and hydrogen are likely to be sufficiently different to support such an unusual type of energy conservation.

*For correspondence: E-mail: Nicolai.Mueller@uni-konstanz.de; Tel. +49-7531-88-3282; Fax +49-7531 88-4047.

Introduction

Formate is formed in classical primary fermentations, such as the mixed-acid fermentation by certain Enterobacteriaceae and by strict anaerobes in pyruvate cleavage by pyruvate formate lyase (Knappe and Wagner, 2001). Formate can be further converted by the same bacteria to $H_2 + CO_2$ by formate hydrogen lyase (Leonhartsberger *et al.*, 2002; Sinha *et al.*, 2015). Other strictly anaerobic bacteria form formate by reduction of CO_2 with low-potential electrons that are typically provided by ferredoxins (Andreesen and Makdessi, 2008). In a similar manner, hydrogen gas can be formed in anaerobic fermentations by hydrogenases (Adams *et al.*, 1980; Peters *et al.*, 2015), most often with ferredoxins as low-potential electron donors. Both formate and hydrogen serve in these reactions as vents for the release of excess electrons, e. g., from the oxidative decarboxylation of 2-oxo acids, to allow ATP synthesis in subsequent substrate-level phosphorylation reactions.

Conversion of reduced products of primary fermentations such as alcohols and fatty acids to methane and CO_2 in methanogenic biomass degradation requires the cooperation of so-called secondary fermenting bacteria with methanogenic partners. The methanogens consume hydrogen and/or formate at low concentration, thus rendering the fermentations of the secondary fermenters energetically feasible, in a symbiotic cooperation called syntrophy (Schink, 1997; McInerney *et al.*, 2008; 2009; Stams and Plugge, 2009; Schink and Stams, 2013). Defined syntrophically cooperating cocultures have been isolated which oxidize ethanol (Bryant *et al.*, 1967), butyrate (McInerney *et al.*, 1979), propionate (Boone and Bryant, 1980), benzoate (Mountfort and Bryant, 1982), or acetate (Zinder and Koch, 1984). Also, the methanogenic degradation of long-chain fatty acids (Roy *et al.*, 1986), branched-chain fatty acids (Stieb and Schink, 1986; 1989), amino acids (Zindel *et al.*, 1988) and glycolic acid (Friedrich *et al.*, 1991) depends on interspecies electron transfer in syntrophic associations. Since the methanogenic partners employed in cultivation of syntrophs could always use both hydrogen and formate as substrates the question remained largely unanswered from the very beginning as to whether really hydrogen or formate acts

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as interspecies electron carrier in these associations (Bryant *et al.*, 1967; McInerney and Wofford, 1992). After introduction of a hydrogen detector system sensitive enough to measure hydrogen at concentrations of 10^{-5} atm (1 Pa) and lower (Schmidt and Seiler, 1970) most studies on energetics, growth kinetics etc. of syntrophic associations were based on measurements of hydrogen pools (Stieb and Schink, 1987; Conrad *et al.*, 1986; Schöcke and Schink, 1997; Seitz *et al.*, 1988; 1989). Nonetheless, also formate was found to act in interspecies electron transfer (Thiele and Zeikus, 1988; Sieber *et al.*, 2014), especially in the syntrophic oxidation of propionate (Boone *et al.*, 1989; Dong *et al.*, 1994a,b). It was speculated that formate might be the preferred electron carrier in the aqueous phase of suspended cultures whereas hydrogen could be an optimal carrier in microbial aggregates such as sewage sludge flocs and sediments, due to its small size and its apolar character (Schink and Stams, 2013). Nonetheless, reports on measurements of formate pools remained scarce, because measurements of formate in liquid media in the micromolar range is difficult, especially in complex reaction mixtures such as sewage sludge contents. It was assumed that hydrogen and formate might be used alternatively or simultaneously as electron carriers, and that enzyme systems such as formate hydrogen lyase (Sinha *et al.*, 2015) or carbon dioxide reductases (Schuchmann and Müller, 2013) would equilibrate these two pools.

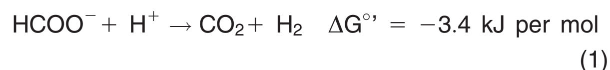
This picture was questioned by recent reports on energy conservation in the conversion of formate to H_2 and CO_2 in anoxic environments (Dolfing *et al.*, 2008; Lim *et al.*, 2012), which indicated that the pool sizes of formate and H_2 might differ by several orders of magnitude to allow for energy conservation in this reaction. Moreover, observations in support of syntrophic interspecies electron transfer by direct electron transfer (Summers *et al.*, 2010) or through magnetite and other iron minerals (Kato *et al.*, 2012; Viggi *et al.*, 2014), cysteine and other thiols (Kaden *et al.*, 2002) or activated charcoal ('biochar', Chen *et al.*, 2014) suggested further routes for interspecies electron transfer and may question the role of hydrogen or formate as electron carriers.

The present review tries to evaluate the relative importance of hydrogen versus formate in natural or technical biogas production and in defined cultures, possible preferences for either carrier under different environmental conditions, and consequences for the assessment of interspecies electron transfer in general.

Topic 1. Calculations

When discussing hydrogen and formate as alternative electron carriers we should consider how the equilibrium of formate conversion to hydrogen plus CO_2 and vice versa is influenced by environmental factors.

Under standard conditions at pH 7.0 (1 M concentrations; gases at 1 atm) the reaction of formate to H_2 plus CO_2 is nearly in equilibrium:



$$HCOO^- + H^+ \rightarrow CO_2 + H_2 \quad \Delta G^{\circ\prime} = -3.4 \text{ kJ per mol} \qquad (1)$$

The Gibbs' free energy is calculated here for gaseous CO_2; if we focus on CO_2 dissolved in water, the ΔG shifts to +4.6 kJ/mol. Since the reaction involves a proton on one side, the ΔG depends on the pH (Fig. 1a, left part) and is more exergonic at lower pH. In the pH range between the two pK values of carbonic acid (at pH 6.3 and 10.4), the reaction partner is bicarbonate rather than CO_2, as follows:

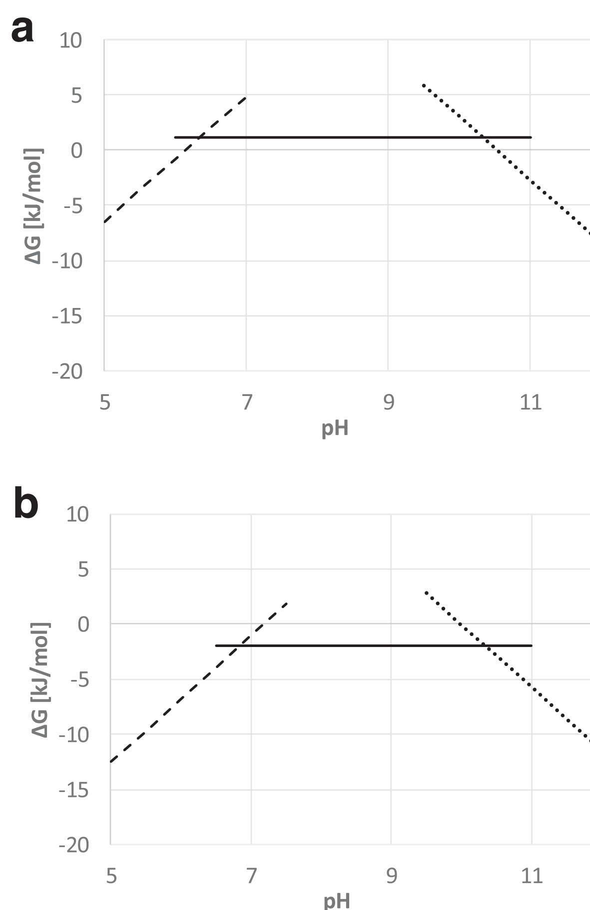


Fig. 1. Impact of pH on Gibbs' free energy of formate-to-hydrogen conversion; with CO_2 (dashed line) HCO_3^- (solid line) and CO_3^{2-} (dotted line) as reaction partner at 25°C, according to equations 1–3 for the respective pH ranges; a) standard conditions, except for pH; b) 'realistic' conditions in a sewage sludge digestor, with reactant activity $1 \cdot 10^4$ for hydrogen, $1 \cdot 10^5$ for formate, 0.2 for CO_2 and $2 \cdot 10^2$ for HCO_3^- and CO_3^{2-}.

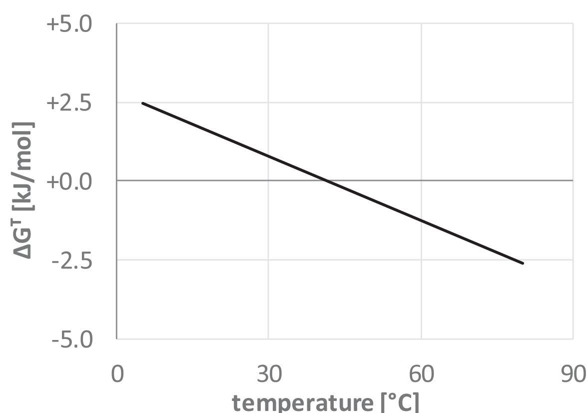
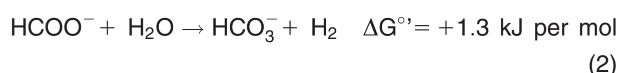
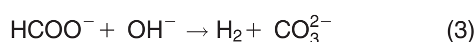


Fig. 2. Impact of temperature on Gibbs' free energy of formate-to-hydrogen conversion with HCO_3^- as reaction partner at pH 7.0.



$$HCOO^- + H_2O \rightarrow HCO_3^- + H_2 \quad \Delta G^{\circ\prime} = +1.3 \text{ kJ per mol} \quad (2)$$

As Fig. 1a (middle part) shows, this reaction is independent of the prevailing pH. Beyond pH 10.4, the reaction partner is mainly the carbonate ion, and the reaction becomes more exergonic with rising pH (Fig. 1a, right part), according to



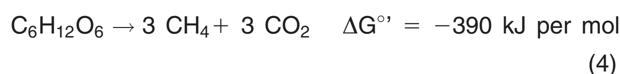
$$HCOO^- + OH^- \rightarrow H_2 + CO_3^{2-} \quad (3)$$

We conclude that, due to the different protonation states of carbonic acid, bicarbonate and carbonate, their reduction to formate follows different reactions (Eq. (1–3)) in the three different pH ranges that are separated by the respective pK values. In the pH range from pH 6.3–10.4, the conversion of formate to hydrogen plus bicarbonate is basically in equilibrium and independent of the prevailing pH; only below and above this range does the pH become relevant for this interconversion. The situation changes only slightly if we take realistic *in situ* conditions rather than standard conditions into consideration (Fig. 1b).

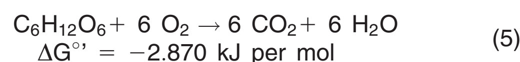
Different from pH, the temperature has only a minor effect on the energetics of formate conversion to hydrogen plus bicarbonate. Over a temperature range from 4°C up to 80°C, the free energy of this reaction changes by only 5 kJ per mol (Fig. 2). Thus, the equilibrium of this reaction is not severely influenced by the reaction temperature, no matter if we look into a cold sediment, a sewage sludge digestor at 35°C or a thermal biogas reactor at 65°C. Obviously, there is no advantage for electron release in the form of hydrogen or formate, respectively, if high or low temperature conditions are considered.

Topic 2. Energetics of interspecies electron transfer

Methane production, be it in a lake sediment or a biogas reactor, starts with the degradation of biomass. Of natural biomass, carbohydrates make up about 50%, followed by proteins (about 30–40%), lipids (10%), nucleic acids etc. For our calculations, we therefore use glucose as a defined molecule that is largely representative of biomass. The conversion of carbohydrates to methane and CO_2 releases in total only 15% of the energy released in aerobic oxidation, as shown below:

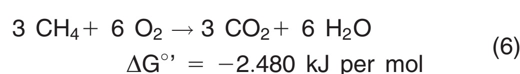


$$C_6H_{12}O_6 \rightarrow 3\ CH_4 + 3\ CO_2 \quad \Delta G^{\circ\prime} = -390 \text{ kJ per mol} \quad (4)$$



$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O \quad \Delta G^{\circ\prime} = -2.870 \text{ kJ per mol} \quad (5)$$

The difference between both is the energy available in aerobic oxidation of methane:



$$3\ CH_4 + 6\ O_2 \rightarrow 3\ CO_2 + 6\ H_2O \quad \Delta G^{\circ\prime} = -2.480 \text{ kJ per mol} \quad (6)$$

Unlike aerobic oxidation, biomass conversion to methane and CO_2 includes at least three different metabolic groups (guilds) of microbes that cooperate intensively with each other. Primary fermenters depolymerize the biomass constituents and ferment sugars, amino acids, glycerol etc. to a mix of fatty acids and alcohols of which acetate, C_1-compounds, and hydrogen can be directly converted to methane by methanogenic archaea. Other fatty acids (longer than C_2) and alcohols (longer than C_1), long-chain fatty acids, branched-chain fatty acids, aromatic acids etc. need to be converted by secondary fermentations to those compounds that can be used by methanogens, i.e. acetate and C_1-compounds. Thus, the small amount of energy available from methanogenic fermentations (equation (4)) has to be shared by several different groups of microorganisms. The major part of the available energy (up to four ATP equivalents, corresponding to about −280 kJ per mol glucose) is consumed by the primary fermenters which take the lion's share of this energy. This was shown nicely by a microcalorimetric incubation study of marine sediment with algal biomass in which the primary fermentations released the major amount of the total energy, followed by small amounts of energy turnover by secondary fermenters, cooperating in this case with sulfate reducers (Graue *et al.*, 2012). Thus, only 1–2 ATP per hexose equivalent are left for the secondary fermenters and the methanogens, thus severely confining their energy budget. Model calculations (see, e.g. Schink, 1997; Schink and Stams, 2013) attribute about 20–30 kJ per reaction run for secondary fermenting bacteria and their methanogenic partners. This value is close to the minimum energy quantum needed for ATP synthesis in a membrane-bound ATPase reaction running at a membrane potential of ca. −200 mV and a

Table 1. Redox potentials of oxidation steps in anaerobic biomass conversion to methane and CO_2.

Oxidation of	Oxidation step	Redox potential at pH 7.0 (mV)	Reference
Glucose	1,3-bisphosphoglycerate/glyceraldehyde-3-phosphate	−350	Thauer *et al.* (1977)
	CO_2 + Acetyl-CoA/Pyruvate	−470	Thauer *et al.* (1977)
Amino acids	2-oxo acid/2-amino acid	−126	Calculated after Thauer *et al.* (1977)
	CO_2 + Acetyl-CoA/Pyruvate	−470	Thauer *et al.* (1977)
Butyrate	CrotonylCoA/ButyrylCoA	−126	Gustafson *et al.* (1986)
		−79	Fink *et al.* (1986)
		−10	Sato *et al.* (1999)
	AcetoacetylCoA/3-oxobutyrylCoA	−250	Thauer *et al.* (1977)
Benzoate	2-3-EnoylCoA/AcylCoA	−126	Gustafson *et al.* (1986)
		−79	Fink *et al.* (1986)
		−10	Sato *et al.* (1999)
	3-oxoacylCoA/3-OH-acylCoA	−250	Thauer *et al.* (1977)
Propionate	Fumarate/Succinate	+30	Thauer *et al.* (1977)
	Oxalacetate/Malate	−172	Thauer *et al.* (1977)
	AcetylCoA + CO_2/Pyruvate	−470	Thauer *et al.* (1977)
Ethanol	Acetaldehyde/Ethanol	−196	Thauer *et al.* (1977)
	AcetylCoA/Acetaldehyde	−370	Thauer *et al.* (1977)
	Acetate/Acetaldehyde	−590	Thauer *et al.* (1977)
Acetate	CO_2/CO	−520	Thauer *et al.* (1977)
	CO_2/formate	−430	Thauer *et al.* (1977)
	CH-THF/CH_2-THF	−300	Blakley *et al.* (1984)
	CH_2-THF/CH_3-THF	−200	Poehlein *et al.* (2012)

stoichiometry of 3–4 H^+ or Na^+ per ATP (Schink, 1997; Schink and Stams, 2013). Needless to say that the available energy varies depending on the substrate supply for the entire methanogenic community, i.e. whether the pools of short-chain fatty acid are at a range of a few micromolar in a meager freshwater lake sediment, or in the range of several millimolar in a biogas reactor. Accordingly, also the pools of hydrogen or formate will vary with the pool sizes of the fermentation intermediates. In Table 1, we compiled a few examples of data from the literature which show that the pools of hydrogen and formate vary depending on the substrate (electron) supply, but that they both vary roughly in parallel. Estimates of Gibbs' free energies of formate to hydrogen conversion according to Eq. (2) in these cases as far the necessary data were available, together with assumptions on prevailing pH and bicarbonate pools gave values around 0 ± 5 kJ per mol. The pools of these two electron carriers, together with that of acetate as the most important short-chain fatty acid, determine the amounts of energy available to the partners in the overall process. Obviously, energy gains below 1 ATP unit require the participation of membrane-bound ion translocation processes, often combined with classical substrate-level phosphorylation and processes described recently as electron bifurcation or electron confurcation (Buckel and Thauer, 2013).

Topic 3. Intracellular electron transport in fermentation processes

In fermentation of sugars to acetyl residues through glycolysis, electrons are released in the oxidation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate at a redox potential of −350 mV, and in oxidation of pyruvate to acetyl-CoA at −470 mV (Table 1). These two electron pairs can easily be released as molecular hydrogen in the oxidation of biomass to acetate as a key intermediate. Further oxidation of acetate is energetically difficult; the typical fate of acetate in methanogenic degradation is its cleavage to $CH_4 + CO_2$. The low-potential electrons of pyruvate oxidation can either be released as molecular hydrogen via ferredoxin as electron carrier, or as formate as known for *Enterobacteriaceae*, with possible subsequent cleavage of formate to H_2 plus CO_2 via formate hydrogen lyase.

Different from 'classical' primary fermenters, the syntrophically glucose-oxidizing *Bacillus stamsii* depends on syntrophic association with hydrogen/formate-utilizing partners because it cannot shift to the formation of reduced fermentation side products such as butyrate or propionate (Müller *et al.*, 2008). It grows optimally with the formate and hydrogen-utilizing *Methanospirillum hungatei*, but does not grow in the presence of the only hydrogen-utilizing *Methanobrevibacter arboriphilus* although both hydrogenase and formate dehydrogenase activities could be identified in cell-free extracts. This bacterium therefore most likely depends on a methanogen that uses formate and hydrogen simultaneously (Müller *et al.*, 2008).

Amino acids as building blocks of proteins are anaerobically degraded via oxidative deamination to the corresponding 2-oxo acids ($E_0{}' = -126$ mV); the further path leads via oxidative decarboxylation to the next shorter fatty acid derivatives, analogous to pyruvate oxidation

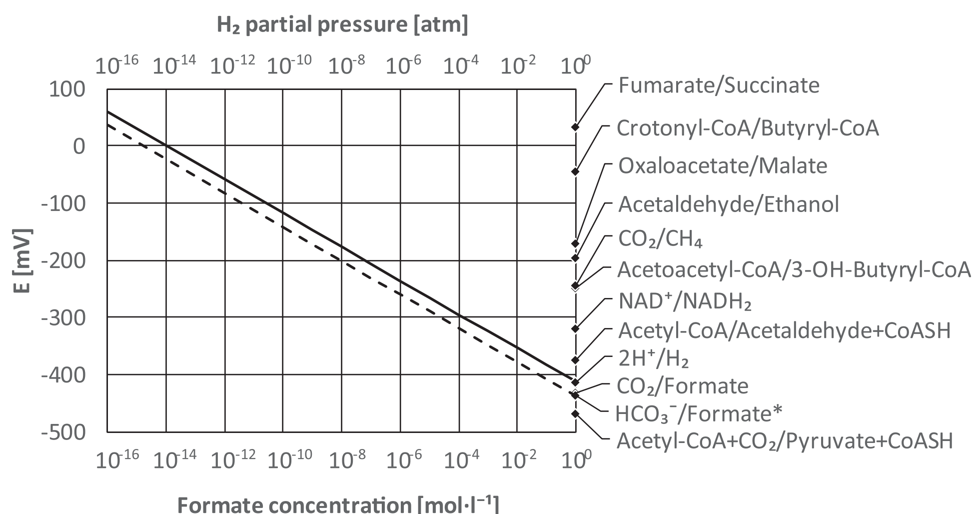


Fig. 3. Redox potentials of the proton/hydrogen pair at various hydrogen partial pressures (solid line) and the bicarbonate/formate pair at 100 mM bicarbonate and various formate concentrations (dashed line) at pH 7.0 and 25°C, in comparison to standard redox potentials of redox reactions of interest in syntrophic oxidation processes.

($E_0' = -470$ mV). Subsequent degradation to methane and CO_2 follows individual pathways, depending on the respective amino acid structure; the energetic problems arising are covered in good approximation in the subsequent treatments of fatty acid degradation.

Syntrophic butyrate oxidation by *Syntrophomonas wolfei* proceeds through beta oxidation and releases two acetate and two pairs of electrons, either as hydrogen or as formate (McInerney and Wofford, 1992). The standard redox potentials of the two oxidation steps are rather different (Table 1): Oxidation of butyryl-CoA to crotonyl-CoA releases electrons at a comparably positive redox potential, i.e. −126 mV, −79 mV or −10 mV, depending on the literature source (see Table 1). In any case, these redox potentials are substantially more positive than that of the proton/H_2 pair or the CO_2/formate pair, even at the low concentrations measured in methanogenic sediments or biogas reactors (Fig. 3). The redox potential of the second oxidation step, 3-hydroxybutyryl-CoA to acetoacetyl-CoA, is defined at −250 mV; this is roughly equivalent to hydrogen partial pressures of 10^{-4} to 10^{-5} atm as typically measured in methanogenic habitats. The NADH electrons gained in this oxidation step go to an enzyme complex that can form either hydrogen or formate (Müller *et al.*, 2009; Schmidt *et al.*, 2013). The electrons derived in butyryl-CoA oxidation are transferred through a flavin-containing EtfAB-carrier and a membrane-bound iron-sulfur protein to a formate dehydrogenase complex oriented outwards which produces formate from CO_2 and external protons, thus using the proton gradient across the membrane to overcome the redox potential shift to free formate (Schmidt *et al.*, 2013). A formate transporter can equilibrate the formate concentrations inside and outside the cell. According to this scheme, formate appears to be the preferred carrier to the methanogenic partner, but formate and hydrogen can be equilibrated by the cytoplasmic formate dehydrogenase/hydrogenase complex. In a parallel study with the same organism, Sieber *et al.* (2015) found preferential induction of hydrogenases over formate dehydrogenases during syntrophic butyrate oxidation. The different outcome of proteomic analysis of the same organism in two different labs may be partially due to minor differences in growth conditions, different supply with trace metals etc. (McInerney and Schink, unpublished). In a recent study, it was confirmed that both hydrogen and formate can be used alternatively as electron carriers by *S. wolfei*, and that hydrogen might be the preferred carrier (Crable *et al.*, 2016).

In order to elucidate a possible preference for either formate or hydrogen as electron carriers to the electron-scavenging partners cocultivation experiments were set up with different partners. Whereas *S. wolfei* appears to use both channels alternatively or simultaneously, *Syntrophospora bryantii* grows optimally with *Methanospirillum hungatei* which uses both carriers whereas cocultures with the mainly formate-oxidizing *Methanobacterium formicicum* were slower, and there was no growth at all with the exclusively hydrogen-consuming *Methanobrevibacter arboriphilus* (Dong *et al.*, 1994a). *S. wolfei* can grow also with the only hydrogen-oxidizing *Methanobrevibacter arboriphilus* but only at a lower rate, indicating that it uses both hydrogen and formate simultaneously as carrier channels (McInerney *et al.*, 1979; 1981).

In contrast to *S. wolfei*, *Syntrophus aciditrophicus* appears to employ in butyrate oxidation a membrane-bound sodium ion-translocating Rnf complex that can transfer electrons from NADH to ferredoxin at the expense of the proton/sodium motive force (McInerney *et al*., 2008). These ferredoxin electrons could be combined with those from butyryl-CoA oxidation in a confurcating manner as described for the Bcd/EtfAB-complex in *Clostridium kluyveri* (Herrmann *et al*., 2008; Li *et al*., 2008). Flavin-dependent electron bifurcation or confurcation has been introduced recently as a means of dismutation of electrons from an intermediate redox potential simultaneously to a lower and a higher redox potential, or *vice versa* (Buckel and Thauer, 2013). Alternatively, the ferredoxin electrons could be released directly as hydrogen or formate by a confurcating hydrogenase or formate dehydrogenase. These suggestions have been derived from genomic and proteomic analysis of *S. aciditrophicus* but have not yet been substantiated by biochemical studies. The biochemical strategy of *S. aciditrophicus* may allow more metabolic flexibility than the one employed by *S. wolfei* which lacks genes for an Rnf complex. Since *S. aciditrophicus* grows not only with butyrate but also with benzoate and can even use benzoate as an electron acceptor in a dismutating type of fermentation a more complex electron transfer system may be advantageous. In any case, anaerobic benzoate degradation proceeds finally through a C_7-dicarboxylic acid derivative (3-hydroxypimelyl-CoA) which is oxidized to acetyl-CoA residues via beta oxidation, thus providing the same problems in electron transfer as in butyrate oxidation (see above; Table 1).

In syntrophic oxidation of propionate, three electron pairs have to be released at rather different redox potentials (Table 1). According to our present knowledge based on biochemical and proteomic evidence with *Syntrophobacter fumaroxidans* and *Pelotomaculum thermopropionicum*, oxidation of succinate to fumarate requires cytochrome b: quinone oxidoreductase and a periplasmic formate dehydrogenase to release these electrons via energy-dependent reverse electron transport to form primarily formate as external electron carrier (De Bok *et al*., 2003; Worm *et al*., 2010; Müller *et al*., 2010; Worm *et al*., 2011a,b). The electrons released in oxidation of malate to oxaloacetate and of pyruvate to acetyl-CoA may be transferred via NADH and ferredoxin to a confurcating hydrogenase. This enzyme type was first described for *Thermotoga maritima* and allows to combine electrons of higher and lower redox potential in hydrogen formation at an intermediate potential (Schut and Adams, 2009). Thus, present evidence indicates that the electrons from succinate oxidation and those from the other two oxidation steps go separate ways, the first ones via formate and the others via hydrogen, to the methanogenic partner. Earlier studies indicated that interspecies formate transfer is an essential means of electron transfer in syntrophic propionate degradation (Dong *et al*., 1994b; Dong and Stams, 1995). Starvation of *S. fumaroxidans* for tungsten impaired propionate transformation rates in methanogenic cocultures, indicating that the tungsten-containing formate dehydrogenase played an essential role in this process (Plugge *et al*., 2009). Alternatively, also a confurcating formate dehydrogenase could deliver the electrons from NADH and from ferredoxin as formate to the partner organism. Oxidation of formate with simultaneous reduction of protons to hydrogen and *vice versa* has been shown in cell suspensions of *S. fumaroxidans* (Stams and Dong, 1995), indicating that also this bacterium can equilibrate these two electron carrier pools.

Although syntrophic ethanol oxidation was the first process of this kind described in the literature its biochemistry has not been understood in detail yet. Since the so-called ‘S-strain’ isolated from ‘*Methanobacillus omelianskii*’ (Bryant *et al*., 1967) has been lost work towards an elucidation of the biochemistry of syntrophic ethanol oxidation has concentrated on *Pelobacter carbinolicus* and *Pelobacter acetylenicus* (Schink, 1984; 1985) which both oxidize ethanol in syntrophic association with *Methanospirillum hungatei* and can be grown in pure culture with acetaldehyde precursors such as acetylene or acetoin, or even with acetaldehyde itself, which all are dismutated to ethanol and acetate (Schmidt *et al*., 2014). In these bacteria, ethanol is oxidized to acetaldehyde by an NAD-dependent alcohol dehydrogenase. For oxidation of acetaldehyde, there are three enzymes available that are all induced during ethanol oxidation, a tungsten-containing acetaldehyde:ferredoxin oxidoreductase and a molybdenum-containing isoenzyme, both forming acetate as product. The molybdenum-dependent acetaldehyde dehydrogenase is formed only under tungsten limitation. Apart from these enzymes, there is an NAD-dependent acetaldehyde dehydrogenase forming acetyl-CoA. Proteomic analysis also indicated the presence of confurcating hydrogenases and formate dehydrogenases which were expressed constitutively and are probably involved in interspecies electron transfer. If both acetaldehyde-oxidizing enzyme systems are actively involved in the overall process, electrons are released at three different redox potentials (Table 1). The mentioned confurcating formate dehydrogenase and hydrogenase may play key roles in this process (N. Müller, unpublished). In cocultures with *M. hungatei*, both hydrogen and formate were measurable at concentrations corresponding to redox potentials of −358 mV and −366 mV respectively (Schmidt *et al*., 2014). These numbers are close to the mean value of the redox potentials of the electron-

releasing steps in ethanol oxidation (Table 1). As the measured pool sizes of hydrogen and formate indicate, both electron carriers are used in this syntrophic culture simultaneously and at equal redox potentials.

Syntrophic oxidation of acetate plays an essential role in methanogenesis at temperatures higher than 50°C, and also at enhanced ammonia concentrations (beyond 1 g NH_3-N · l^{-1}; i.e. ca. 70 mM; Moestedt *et al*., 2016). The pathway of syntrophic acetate oxidation appears to be a reversal of the Wood-Ljungdahl pathway of homoacetogenesis (Schnürer *et al*., 1997; Hattori *et al*., 2005). According to its genome analysis, *Thermacetogenium phaeum* contains a formate hydrogen lyase complex as well as three further formate dehydrogenases and three hydrogenases, but no cytochromes and no Rnf complex (Oehler *et al*., 2012) whereas the mesophilic *Tepidanaerobacter acetatoxydans* contains hydrogenases and an Rnf-complex but no formate dehydrogenase or F_1F_O ATPase (Müller *et al*., 2015). Thus, the energy metabolism of these two types of syntrophic acetate oxidizers may be organized in slightly different ways. The redox potentials of the oxidation steps in the reversed Wood-Ljungdahl pathway are again rather different (Table 1). The comparably 'positive' electrons of methyl-tetrahydrofolate oxidation may find their way to hydrogen or formate via confurcation with low-potential electrons from CO or formate oxidation, but reliable biochemical studies on these processes are lacking so far. Formate appears as a free intermediate in this pathway and can be released directly as an electron carrier to the partner organism rather than being oxidized to CO_2; this may explain why *T. acetatoxydans* can run this metabolism without a formate dehydrogenase enzyme.

Beyond formate and hydrogen, also other carriers have been discussed through the recent years to transfer electrons between syntrophic partners. Based mainly on studies on extracellular electron transfer to iron oxides, humic acid analogs such as anthraquinone disulfonate (Lovley *et al*., 1998), activated carbon and biochar (Liu *et al*., 2012; Chen *et al*., 2014) and various iron oxides (Kato *et al*., 2012; Viggi *et al*., 2014; Zhou *et al*., 2014) have proven to enhance interspecies electron transfer. Moreover, a cystine/cysteine and a polysulfide/sulfide cycle were found to transfer electrons in laboratory cocultures of *Geobacter sulfurreducens* and *Wolinella succinogenes* (Kaden *et al*., 2002). Last but not least, there is even evidence of direct electron transfer between syntrophic partners, either via direct interspecies electron transfer (Shrestha *et al*., 2013; Rotaru *et al*., 2014a,b; Li *et al*., 2015), or via intercellular electrically conductive pili, so-called nanowires (Shresta and Rotaru, 2014). In all these cases, be it defined cocultures or undefined enrichments or digestor contents, a substantial increase of interspecies electron transfer was observed upon carrier addition. For a critical assessment of these observations, one has to keep in mind that the majority of these electron carriers have standard redox potentials of −200 mV and higher. If iron oxides such as ferrihydrite ($E_0' = -100$ to $+100$ mV) or humic compounds ($E_0' = -200$ to $+300$ mV; Straub *et al*., 2001) are to act as terminal acceptors, the entire electron freight of organic matter oxidation can be transferred through such carriers. On the other hand, coupling interspecies electron transfer to methanogenesis requires a sufficiently low redox potential: the redox couple CO_2/CH_4 has an $E_0' = -244$ mV; if we leave a minimum amount of energy for the methanogens for their own ATP supply, electrons have to be delivered by the partner at an average E_0' of −270 mV. If part of the electron transfer to methanogenesis proceeds through anthraquinone disulfonate ($E_0' = -184$ mV), humic substances or ferrihydrite, a substantial further part of electrons has to be delivered through other carriers at lower redox potential, e.g. hydrogen or formate, to secure a sufficient energy supply for the methanogens. It should be noted at this point that some iron oxides such as goethite, hematite or magnetite have redox potentials E_0' of −274, −287, and −314 mV (Straub *et al*., 2001) which would be sufficiently low to cover the energetical needs of the methanogens.

Direct interspecies electron transfer from cell to cell or via electroconductive pili requires close spatial associations between the partners involved. This is easily conceivable for methanogenic communities growing slowly in a stable environment such as a lake sediment. In technical settings such as sewage digestors, biogas reactors etc. which undergo intensive mixing, interspecies electron transfer is achieved probably much more efficiently by diffusive electron carriers such as hydrogen or formate. It is interesting to note in this context that all classical enrichments for syntrophic fatty acid- or alcohol-oxidizing cultures cooperate via hydrogen or formate transfer, and that other forms of interspecies electron transfer have been observed so far either in non-defined enrichments that were supplied with external carriers, or in artificially constructed cocultures.

Topic 4. Formate and hydrogen equilibria

If we calculate the redox potentials of formate oxidation at various formate concentrations and compare them with those of hydrogen at various pressures it turns out that 10^{-6} M formate corresponds to a hydrogen partial pressure of 10^{-5} atm or 10 ppm (Fig. 3). This calculation is based on an assumed bicarbonate concentration of 0.1 M which is roughly representative of bicarbonate concentrations in lake sediments (50–100 mM), growth media (20–50 mM), and biogas reactors (200–400 mM).

Table 2. Pools of hydrogen, formate, and acetate in natural habitats, bioreactors and defined co-cultures. * Unpublished results from the authors' lab, Methods according to Montag and Schink (2016).

Type of habitat	Substrate	H_2 (ppm)	Formate (μM)	Acetate (mM)	Reference
Lake Mendota sediment	-	30–40	n. d.	0.032	Conrad *et al.* (1986)
Fen soil slurry (pH 4.5)	Formate pulses	1000	ca. 5000	20	Hunger *et al.* (2011)
Lab reactor	Sucrose	3000	2000–3000	7	Pauss *et al.* (1990)
Chemostat, defined coculture *Desulfovibrio/Methanospirillum*	Lactate	30 000–40 000	100–300	28	Meyer *et al.* (2013)
Sewage sludge	-	270	n. d.	0.36	Conrad *et al.* (1986)
Sewage sludge	-	44	10	10	Montag and Schink (2016)
Biogas reactor	Maize silage + cow manure	10–20	1	0.2	Montag and Schink (2016)
Biogas reactor (high ammonia content, ca. 300 mM)	Maize silage, cow manure + chicken feces	30	11	10	Montag and Schink (2016)
Biogas reactor + high load of Glucose	Maize silage + cow manure	48	52	75.7	Montag and Schink, unpubl.*
Biogas reactor + high load of Urea	Maize silage + cow manure	23	350	18.1	Montag and Schink, unpubl.*

Table 2 summarizes pool sizes of hydrogen and formate in a variety of natural and man-made microbial communities. According to Table 2, the pools of hydrogen (3000 ppm) and formate (2000–3000 μM) in a lab reactor fed with sucrose are not exactly in thermodynamic equilibrium but leave an energy gap of about 6 kJ per mol (Pauss *et al*., 1990). The same applies to a defined chemostat coculture of *Desulfovibrio vulgaris* and *Methanococcus maripaludis* fed with lactate, thus reflecting a slight imbalance between both pools which most likely is associated with a higher preference for hydrogen of the syntrophic methanogen (Meyer *et al*., 2013). Also biogas reactor contents studied during 'normal' operation and in stress situations of excess sugar or urea feeding exhibited hydrogen and formate concentrations that were more or less energetically equivalent.

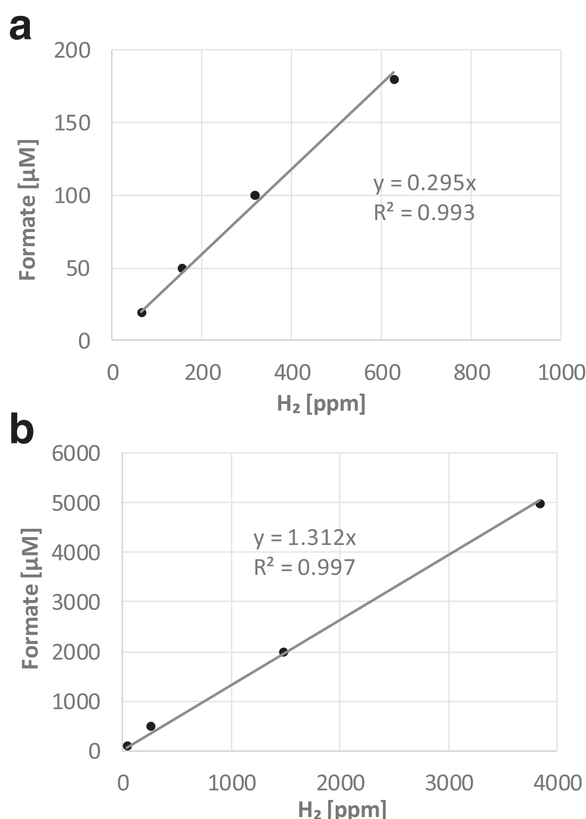


Fig. 4. Ratios of formate and hydrogen reached in (a) a cell suspension of *Acetobacterium woodii* or (b) biogas reactor digest after incremental formate additions. The calculated Gibbs' free energies after equilibration (30 min reaction time each) were −3.0 kJ per mol (*A. woodii*, OD 6.8, 30°C, pH 7.8) and −5.7 kJ per mol (biogas reactor digest (40°C, pH 8.0).

In defined cultures, formate-to-hydrogen ratios can differ from the expected values. To examine this problem on a broader basis, we measured formate-to-hydrogen conversion equilibria with two different bacteria. The carbon dioxide reductase of *Acetobacterium woodii* has been described to interconvert both reactants until thermodynamic equilibrium is reached (Schuchmann and Müller, 2013). We reexamined this claim with cell suspensions of this bacterium and could confirm it at least for formate conversion to hydrogen (Fig. 4a). In the opposite direction, the phase transfer of hydrogen from the gas phase to the aqueous phase at low partial pressure turned out to be a major kinetic obstacle for such measurements. A similar situation was observed with biogas reactor digest (Fig. 4b) indicating that in such complex microbial communities the pools of formate and hydrogen tend to reach a thermodynamic equilibrium.

In similar experiments, we measured and calculated hydrogen and formate equilibria also with cell suspensions and cell-free extracts of *Escherichia coli* (Table 3). Depending on whether HCO_3^- or CO_2 was used for calculation, reaction enthalpies ranged from −8.1 to −16.3 kJ per mol, indicating that this part of the reaction energy was not exploited (Table 3). Other authors demonstrated hydrogen production from formate with purified formate hydrogenlyase of *E. coli* (Pinske and Sargent, 2016). Here, 15.7 mM formate was added to the purified enzyme in the reaction chamber of a hydrogen-sensing

Table 3. Reaction enthalpies of formate-to-hydrogen conversion calculated from equilibrium concentrations of formate and hydrogen in experiments with dense cell suspensions or cell-free extracts of *Escherichia coli*. Shown are mean values and standard deviations of $n = 3$.

	Initial concentration formate [mM]	Final concentration formate [mM]	Final H_2 partial pressure [ppm]	$\Delta G'$ (HCO_3^-) [kJ per mol]	$\Delta G'$ (CO_2) [kJ per mol]
Cell suspension	1	0.9 ± 0.1	284.3 ± 38.6	−14.3 ± 0.3	−8.1 ± 0.3
	0.5	0.6 ± 0.2	128.7 ± 35.8	−15.0 ± 1.8	−8.8 ± 1.8
Cell-free extract	0.5	0.4 ± 0.2	84.7 ± 7.5	−16.3 ± 3.0	−8.6 ± 1.3

E. coli K12 strain MG1655 was grown anaerobically with 10 mM glucose and 0.05% yeast extract for 5 h at 30°C in non-reduced freshwater medium (Widdel *et al.*, 1983). Cells were washed three times with a three fold volume of potassium phosphate buffer (50mM, pH 7.5). The cell suspension was adjusted to an OD_{600} of 6 or 1 and kept on ice until further use. The gas phase of the cell suspension was exchanged under stirring by applying vacuum and then flushing with a mixture of N_2/CO_2 (80%/20%) three times. The cell suspension was equilibrated to 30°C and the experiment was started by adding formate to a start concentration of 500 μM or 1000 μM respectively. To prepare cell free extract, the cells were opened by three times passing through an anoxic French press (Schmidt *et al.*, 2013) and cell debris was removed by centrifuging for 5 min at 11 300*g*. The gas phase of the CFE was exchanged against N_2/CO_2 (80%/20%) as explained above. Different volumes (100–300 μL) of the CFE were added to 2 mL of 50 mM potassium phosphate buffer pH 7.5 with 3 mM DTT. The experiment was carried out as described above. Formate samples of start and end point were analyzed by HPLC according to Montag and Schink (2016). Hydrogen formation was monitored with a reductive gas chromatograph as described by Montag and Schink (2016).

Clark electrode and maximal production of around 600 nmol hydrogen in 1.7 ml volume was observed, with a final formate concentration of 14.9 mM. The final concentrations of formate, hydrogen, and CO_2 (calculated from the consumed formate) were 14.9 mM, 353 μM and 840 μM respectively (Pinske and Sargent, 2016). From these values and the standard Gibbs' free reaction energy of −3.4 kJ per mol (Eq. (1)) we calculated a remaining reaction enthalpy of -30.2 kJ per mol. Assuming that equal amounts of hydrogen and CO_2 were produced from 840 μM consumed formate, the reaction enthalpy changes to −28 kJ per mol. Values for reaction enthalpies always vary depending on the assumed parameters, however, we never observed values for *E. coli* formate hydrogenlyase that were close to $\Delta G' = 0$, which would be a prerequisite for the reaction equilibrium. Formate hydrogenlyase of *E. coli* has a rather low substrate affinity for formate, with a K_m value of 26 mM (Axley and Grahame, 1991). This low substrate affinity could explain why formate-to-hydrogen ratios in anaerobic cultures of *E. coli* are often not balanced. The enzyme has probably not evolved to exploit the complete reaction energy as it solely serves as a pH-regulating enzyme at high formate concentrations. Although the enzyme is membrane-associated there is no indication that formate oxidation is coupled to proton translocation across the membrane, and hence it probably does not conserve energy (Pinske and Sargent, 2016), different from the formate-oxidizing system of *Thermococcus onnurineus* (Lim *et al.*, 2012; see below).

In syntrophic associations, secondary fermenting microorganisms need to be metabolically flexible to be prepared for changing environmental conditions, i. e. the extinction or the emergence of a hydrogen- or formate-scavenging partner organism, as suggested for the amino acids oxidizing *Eubacterium acidaminophilum* (Zindel *et al.*, 1988; Graentzdoerffer *et al.*, 2003). Therefore, these organisms have to provide enzyme systems to allow them to switch quickly between formate and hydrogen production, or to metabolically link the pools of formate and hydrogen at low concentration. Such an enzyme system is the formate dehydrogenase of *Eubacterium acidaminophilum* which obviously links formate oxidation to hydrogen production and *vice versa*, thus representing a simple type of formate hydrogen lyase (Graentzdoerffer *et al.*, 2003). Similar enzymes in *Syntrophomonas wolfei* or *Pelobacter acetylenicus* may act in a similar manner. On the other hand, production of formic acid by *E. coli* during mixed acid fermentation leads to a pH decrease of the growth medium, especially when the culture reaches stationary phase (Pinske and Sargent, 2016). *E. coli* can compensate this effect by expressing formate hydrogen lyase which oxidizes formate and transfers electrons to protons to form hydrogen as a final product (Pinske and Sargent, 2016). Due to its low formate affinity, the conversion of formate to hydrogen plus CO_2 by this enzyme is kinetically rather than energetically limited. Thus, the primary functions of formate-to-hydrogen converting enzyme systems depend on the ecology and physiology of the respective organism.

These findings give rise to the question whether it is possible at all to use formate and hydrogen pool sizes to draw general conclusions on the observed ecosystem. Thermodynamic calculations could potentially help to understand whether formate-to-hydrogen conversion in an observed system has an energy-conserving purpose or can be attributed to secondary metabolism reactions such as pH regulation. Other than that, complex microbial communities can exhibit numerous causes of a shift of the formate-to-hydrogen ratio, and other metabolites such as methane and acetate have to be considered as well. Especially biogas reactors underly temporal discontinuities depending on the feeding

rhythm and the mode of mixing. Insufficient mixing may cause spatial inhomogeneities also at the microscale and may raise questions towards the applicability of bulk pool size measurement results for an energetic assessment of microbial interactions at small scale in general (Pauss and Guiot, 1993). At least for the biogas reactor digest documented in Fig. 4b, we can state that formate and hydrogen pools tend to reach thermodynamic equilibrium.

Topic 5. Formate fermentation to $H_2 + HCO_3^-$, an energetically feasible basis for life?

Can the conversion of formate to $H_2 + HCO_3^-$ sustain microbial growth? As shown above, the reaction of formate to $H_2 + HCO_3^-$ at neutral pH is nearly at thermodynamic equilibrium and even slightly endergonic (+1.3 kJ per mol, equation (2)). Microbial life requires a minimum amount of about 15 – 20 kJ per mol reaction to maintain a charged cytoplasmic membrane and, with this, to synthesize ATP (Schink, 1997; Schink and Stams, 2013). With a sufficiently high activity difference between formate and hydrogen (e.g. formate at 10 mM, H_2 at 10^{-4} atm) conditions can be established in the laboratory that could supply sufficient energy for an energy metabolism based on this reaction. In 2008, Dolfing *et al.* (2008) showed microbial growth of two cocultures, a mesophilic and a thermophilic one, which both grew essentially by conversion of formate to methane with hydrogen as an intermediate. The authors concluded that the first organism in the respective cocultures grew by conversion of formate to hydrogen, and they also provided a metabolic scheme for charge separation by such a metabolism with an externally oriented formate dehydrogenase and an internally oriented hydrogenase. Nonetheless, growth was documented only for the cocultures, not for the first organism in the respective cultures, and it remains unclear whether this organism really obtains energy from the conversion of formate to hydrogen.

In a further study, the thermophilic archaeon *Thermococcus onnurineus* was shown to be able to grow with formate in pure culture at 80°C, forming hydrogen as the only reduced product (Kim *et al.*, 2010). The energetic situation of this organism during this cultivation was calculated to shift from −20 kJ per mol to −8 kJ per mol (Kim *et al.*, 2010; Lim *et al.*, 2012). The bacterium contains a Na^+-dependent ATPase, a Na^+/H^+ antiporter module as well as a membrane-bound formate dehydrogenase and a membrane-bound hydrogenase. According to the authors' interpretation, formate dehydrogenase and hydrogenase together establish a proton gradient across the membrane which is translated into a sodium ion gradient which finally drives ATP synthesis (Lim *et al.*, 2014). It has to be emphasized that at 80°C the conversion of formate to H_2 and bicarbonate is by 3.5 kJ per mol more exergonic than under standard conditions (see Fig. 2). Moreover, organisms like *T. onnurineus* have been isolated from hydrothermal vents which release, among others, formate at millimolar concentrations (Schrenk *et al.*, 2013). Under these specific conditions, this unusual life style appears to be possible and energetically favorable. Whether formate conversion to hydrogen plus bicarbonate or CO_2 can sustain microbial life also in other anoxic environments remains still to be elucidated.

In the previous section, the possibility was briefly discussed that *E. coli* can couple formate hydrogenlyase activity to proton translocation. It was demonstrated earlier that membrane vesicles of anaerobically grown *E. coli* cells generate a membrane potential upon addition of formate (Hakobyan *et al.*, 2005). Other authors discussed the possibility that due to its membrane association, formate hydrogenlyase of *E. coli* might translocate protons across the membrane, and they considered it likely that even if being rudimentary in *E. coli*, this enzyme could be an evolutionary leftover from ancient microorganisms that lived at hydrothermal vents (McDowall *et al.*, 2014). After the hypothetical 'minimum energy quantum' for microbial life has been decreased slowly from −20 kJ (Schink, 1997) to about −10 kJ per mol (Spahn *et al.*, 2015; Lever *et al.*, 2015) the conditions for sustaining sufficient energy for microbial life by this reaction may become easier to achieve. Nonetheless, numerous anaerobic bacteria contain enzymes such as carbon dioxide reductase (Schuchmann and Müller, 2013) or similar enzymes like the hydrogenase/formate dehydrogenase complexes in syntrophic butyrate or ethanol-oxidizing bacteria. These enzymes are known to equilibrate the pools of formate and hydrogen and, with this, annihilate the chances for growth by formate-dependent hydrogen production in mixed microbial communities. That formate and hydrogen pools are quickly being equilibrated also in a natural environment has been shown recently for the microbial communities present in a methane-emitting slightly acidic fen (Hunger *et al.*, 2016).

Conclusions

Although simultaneous measurements of hydrogen and formate pools in methanogenic environments are scarce the evidence at hand from published and unpublished work suggests that both pools are energetically basically equivalent, at least in sewage sludge and biogas reactors. Transient disequilibria may arise with feeding imbalances. For secondary fermenting processes, this means that electron transfer to syntrophic partner organisms, e.g. methanogens, can use either carrier system or both

together simultaneously, and that the electron consumer has no specific advantage from using either carrier exclusively. Hydrogen pools in biogas reactors, even if not always in perfect equilibrium with the formate pool, may still provide a reliable measure of the electron availability, indicating whether the capacity of electron utilizers in such a system is sufficient. Such methanogenic communities typically do not provide metabolic niches for energy conservation by conversion of formate to hydrogen and bicarbonate. An exception in this respect are microbes living at thermal vents under conditions of high formate concentration and low hydrogen pressure where a biochemical apparatus for exploitation of this small energy potential could evolve.

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