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Minireview

Syntrophic butyrate and propionate oxidation processes: from genomes to reaction mechanisms

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Summary

In anoxic environments such as swamps, rice fields and sludge digestors, syntrophic microbial communities are important for decomposition of organic matter to CO_2 and CH_4. The most difficult step is the fermentative degradation of short-chain fatty acids such as propionate and butyrate. Conversion of these metabolites to acetate, CO_2, formate and hydrogen is endergonic under standard conditions and occurs only if methanogens keep the concentrations of these intermediate products low. Butyrate and propionate degradation pathways include oxidation steps of comparably high redox potential, i.e. oxidation of butyryl-CoA to crotonyl-CoA and of succinate to fumarate, respectively, that require investment of energy to release the electrons as hydrogen or formate. Although investigated for several decades, the biochemistry of these reactions is still not completely understood. Genome analysis of the butyrate-oxidizing *Syntrophomonas wolfei* and *Syntrophus aciditrophicus* and of the propionate-oxidizing *Syntrophobacter fumaroxidans* and *Pelotomaculum thermopropionicum* reveals the presence of energy-transforming protein complexes. Recent studies indicated that *S. wolfei* uses electron-transferring flavoproteins coupled to a menaquinone loop to drive butyryl-CoA oxidation, and that *S. fumaroxidans* uses a periplasmic formate dehydrogenase, cytochrome *b*: quinone oxidoreductases, a menaquinone loop and a cytoplasmic fumarate reductase to drive energy-dependent succinate oxidation. Furthermore, we propose that homologues of the *Thermotoga maritima* bifurcating [FeFe]-hydrogenase are involved in NADH oxidation by *S. wolfei* and *S. fumaroxidans* to form hydrogen.

Introduction

In anoxic environments such as swamps, rice paddy fields and intestines of higher animals, methanogenic communities are important for decomposition of organic matter to CO_2 and CH_4 (Schink and Stams, 2006; Mcinerney *et al.*, 2008; Stams and Plugge, 2009). Moreover, they are the key biocatalysts in anaerobic bioreactors that are used worldwide to treat industrial wastewaters and solid wastes. Different types of anaerobes have specified metabolic functions in the degradation pathway and depend on metabolite transfer which is called syntrophy (Schink and Stams, 2006). The study of syntrophic cooperation is essential to understand methanogenic conversions in different environments (Mcinerney *et al.*, 2008). The most difficult step in this degradation is the conversion of short-chain fatty acids such as propionate and butyrate. Under standard conditions (P_{H2} of 1 atm, substrate and product concentrations of 1 M, temperature 298°K), propionate and butyrate oxidation to H_2, formate and acetate are endergonic reactions (Table 1). In anoxic environments, methanogenic *Archaea* maintain low H_2, formate and acetate concentrations which make propionate and butyrate degradation feasible (Stams and Plugge, 2009). Syntrophic propionate and butyrate oxidations involve energy-dependent reactions that are biochemically not fully understood. However, recently several novel reactions were discussed to perform energy transformation in other bacteria. These reactions will be summarized in this report with respect to their possible implications in syntrophic fatty acid oxidation. Moreover, the genomes of two propionate degraders (*Syntrophobacter fumaroxidans* and *Pelotomaculum thermopropionicum*) and two butyrate degraders (*Syntrophomonas wolfei* and *Syntrophus aciditrophicus*) have been sequenced (Mcinerney *et al.*, 2007; Kosaka *et al.*, 2008).

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Table 1. Standard free reaction enthalpies of fatty acid oxidation and methane production.

Reaction		$\Delta G^{0\prime}$ (kJ per reaction)
$Propionate^- + 2\ H_2O \rightarrow Acetate^- + CO_2 + 3\ H_2$	Eq. 1	+76.0
$Propionate^- + 2\ H_2O + 2\ CO_2 \rightarrow Acetate^- + 3\ HCOO^- + 3\ H^+$	Eq. 1a	+65.3
$Butyrate^- + 2\ H_2O \rightarrow 2\ Acetate^- + H^+ + 2\ H_2$	Eq. 2	+48.3
$Butyrate^- + 2\ H_2O + 2\ CO_2 \rightarrow 2\ Acetate^- + 2\ HCOO^- + 2\ H^+$	Eq. 2a	+38.5
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	Eq. 3	–131.7
$4\ HCOO^- + 4\ H^+ \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	Eq. 4	–144.5
$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	Eq. 5	–36

Values calculated from the standard free formation enthalpies of the reactants at a concentration of 1 M, pH 7.0 and T = 25°C according to Thauer and colleagues (1977).

Based on genome analysis we propose that novel energy-transforming reactions are involved in syntrophic butyrate and propionate degradation.

Topic 1. Known energy-conserving mechanisms in syntrophic butyrate and propionate degradation

Butyrate degradation

Butyrate oxidizers known to date belong to two groups of bacteria within the family *Syntrophomonadaceae* and the order *Syntrophobacterales*. Formerly classified as *Clostridia*, the members of the family *Syntrophomonadaceae* have been reassigned to a new family within the order *Clostridiales*, based on their 16S rRNA sequence (Zhao *et al*., 1993). Members of this family are *S. wolfei*, *Syntrophomonas bryantii* [formerly *Syntrophospora bryantii* (Wu *et al*., 2006)], *Syntrophomonas erecta*, *Syntrophomonas curvata*, *Syntrophomonas zehnderi* and *Thermosyntropha lipolytica*.

The second group of syntrophic butyrate degraders belongs to the *Syntrophobacterales*, an order of the deltaproteobacteria subdivision. Organisms of this group are *S. aciditrophicus* and *Syntrophus buswellii*. Several other *Syntrophus* strains such as *Syntrophus gentianae* are able to oxidize benzoate or other aromatic compounds syntrophically but these processes are not considered in this article. Remarkably, all these organisms are restricted to the use of saturated or unsaturated fatty acids. Alternative substrates or alternative electron acceptors to grow these bacteria in pure culture have not been found yet for these two groups of butyrate-oxidizing bacteria.

In all known butyrate-oxidizing bacteria, the beta-oxidation pathway is used (Wofford *et al*., 1986; Schink, 1997; Mcinerney *et al*., 2007). First, butyrate is activated to butyryl-CoA with acetyl-CoA by a CoA transferase. Further oxidation proceeds via crotonyl-CoA and 3-hydroxybutyryl-CoA to acetoacetyl-CoA which is cleaved to two acetyl-CoA moieties. One of these is invested in butyrate activation, and the other one forms ATP via phosphotransacetylase and acetate kinase (Wofford *et al*., 1986). Electrons are released in the oxidation of butyryl-CoA to crotonyl-CoA and in the oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA at –250 mV (Gustafson *et al*., 1986). As the standard midpoint redox potentials of these reducing equivalents are too high for reduction of protons to form H_2 [–414 mV (Thauer *et al*., 1977; Schink, 1997)], it was postulated that an energy-dependent reversed electron transport is required to overcome the redox potential difference (Thauer and Morris, 1984). The partner organism keeps the hydrogen partial pressure low, thus raising the redox potential of proton reduction to a level around –300 mV (Schink, 1997). The butyrate-oxidizing organism has to sacrifice part of the gained ATP to shift electrons to this redox potential, and the remaining ATP can be used for biosynthesis and growth. As such fractional amounts of ATP cannot be provided by substrate level phosphorylation such energy transformations have to be coupled to the cytoplasmic membrane (Thauer and Morris, 1984). Indeed, hydrogen production from butyrate has been shown to be sensitive to the protonophore CCCP and the ATPase inhibitor DCCD, thus providing evidence for participation of a transmembrane proton potential (Wallrabenstein and Schink, 1994). However, the underlying biochemical mechanisms remained enigmatic until the completion of the genome sequence of *S. aciditrophicus* (Mcinerney *et al*., 2007). It was stated that electrons released in butyryl-CoA oxidation are transferred to components of the membrane where they reduce NAD^+ to NADH in an endergonic manner, e.g. through an *rnf*-coded oxidoreductase, and the necessary energy would be supplied by a sodium ion gradient which in turn is provided by ATP-dependent proton efflux and a sodium/proton antiporter. In *S. wolfei*, however, a different reaction mechanism has to be active since the genome of this bacterium does not contain *rnf* genes which will be discussed later in this review (Müller *et al*., 2009).

Propionate degradation

Several bacterial strains are known to degrade propionate in syntrophic association with methanogens:

S. fumaroxidans, *S. wolinii*, *S. pfennigii*, *S. sulfatireducens*, *P. thermopropionicum, P. schinkii, P. propionicicum, Smithella propionica* and *Desulfotomaculum thermobenzoicum* ssp. *thermosyntrophicum.* These bacteria belong to the *Syntrophobacterales*, an order of the deltaproteobacteria subdivision, and to the family *Peptococcaceae* within the order *Clostridiales*. *Smithella propionica* converts propionate through a dismutating pathway to acetate and butyrate after which butyrate is oxidized to acetate (de Bok *et al.*, 2001). All other known syntrophic propionate degraders oxidize propionate to acetate plus CO_2. They use the methylmalonyl-CoA pathway which generates per molecule propionate one ATP via substrate level phosphorylation and three electron pairs by: (i) oxidation of succinate to fumarate ($E^{\circ\prime} = +30$ mV), (ii) oxidation of malate to oxaloacetate ($E^{\circ\prime} = -176$ mV), and (iii) pyruvate conversion to acetyl-CoA and CO_2 ($E^{\circ\prime} = -470$ mV) (Fig. 2). The latter step can easily be coupled to proton reduction ($E^{\circ\prime} = -414$ mV) or CO_2 ($E^{\circ\prime} = -432$ mV) reduction (Thauer *et al.*, 1977) via ferredoxin, as anaerobic bacteria generally contain pyruvate : ferredoxin oxidoreductases (Chabrière *et al.*, 1999).

Oxidation of succinate and malate with protons would require hydrogen partial pressures of 10^{-15} and 10^{-8} atm respectively (Schink, 1997). Thauer and Morris (1984) and Schink (1997) proposed a reversed electron transport mechanism. The hydrolysis of 2/3 ATP coupled with a transmembrane import of two protons would make succinate oxidation energetically possible. Later, van Kuijk and colleagues (1998) proposed that this reaction is analogous to that involved in fumarate respiration by *Wolinella succinogenes*. This bacterium generates a transmembrane proton gradient by periplasmic hydrogen or formate oxidation coupled to cytoplasmic fumarate reduction via cytochromes and a menaquinone loop (Kröger *et al.*, 2002). In *S. fumaroxidans*, fumarate reductase and succinate dehydrogenase activity are membrane bound. Hydrogenase and formate dehydrogenase activity are found in the periplasmic space loosely attached to the membrane, and cells contain cytochrome *c* and *b* and menaquinone-6 and -7 as possible electron carriers (van Kuijk *et al.*, 1998). *Syntrophobacter fumaroxidans* appears to gain around 2/3 ATP per mol fumarate if H_2 or formate is oxidized with fumarate. It was suggested that this mechanism in reverse could reduce periplasmic protons with the energy-dependent cytoplasmic succinate oxidation.

Malate oxidation to oxaloacetate ($E^{\circ\prime} = -176$ mV) is coupled to NAD^+ reduction ($E^{\circ\prime} = -320$ mV) (Van kuijk and Stams, 1996). Yet, the exact mechanism of NADH oxidation and terminal reduction of protons and/or CO_2 in *S. fumaroxidans* remains unclear and deserves further investigation.

Topic 2. Mechanisms for energy conservation in anaerobic microorganisms

Electron transport phosphorylation

Electron transport phosphorylation is the most important energy-conserving mechanism in organisms that reduce external electron acceptors such as oxygen, nitrate, sulfate, etc. (Richardson, 2000). In most cases, the membrane-bound NADH dehydrogenase (complex I) oxidizes NADH with quinones in the membrane while translocating protons into the periplasmic space via a transmembrane proton pump (Richardson, 2000). The electrons are transferred further to the respective terminal acceptor via cytochromes.

Protons can be translocated to the periplasmic space by at least two possible mechanisms. The first one includes the translocation of protons or sodium ions through the transmembrane proton channel of an NADH dehydrogenase (Richardson, 2000). The second mechanism involves a redox loop and is supposed to be the most common way of proton translocation in bacteria (Richardson, 2000). Here, isoprenoid quinones within the membrane are reduced by the membrane-integral domain of the electron-donating enzyme, together with protons. The reduced quinone diffuses laterally through the membrane to the membrane domain of the accepting enzyme where electrons are transferred to an electron acceptor, and the protons are released at the opposite side of the membrane. An example is the redox loop of the formate dehydrogenase FDH-N coupled to nitrate reductase in *Escherichia coli* (Jormakka *et al.*, 2002). During anaerobic growth, formate is oxidized in the periplasm by FDH-N while protons are transferred to menaquinones, together with the electrons released in formate oxidation. Subsequently, menaquinol is oxidized at the membrane domain of the nitrate reductase, releasing protons to the periplasm while electrons are transferred to nitrate to form nitrite at the cytoplasmic side of the membrane (Jormakka *et al.*, 2002).

The smallest quantum of energy in biology

Since only small amounts of chemical energy can be transformed in syntrophic oxidation processes (Table 1), energy has to be efficiently conserved. Thauer and colleagues (1977) and Schink (1997) calculated that the minimal cost of synthesis of one ATP is 60 kJ per mol. Syntrophically fermenting bacteria such as butyrate and propionate oxidizers have to invest part of their ATP to create a proton gradient. For a long time it was thought that three protons are imported for synthesis of one ATP and thus the smallest quantum of energy that can be converted into ATP is in the range of 20 kJ per mol. However, Nakanishi-Matsui and Futai (2008) documented

that the number of protons translocated is determined by the number of membrane-integral c-subunits of the ATP synthase which varies between different microorganisms. ATP synthases of yeast and *Enterococcus hirae* harbour 10, while *Ilyobacter tartaricus*, *Methanopyrus kandleri* and chloroplasts harbour 11, 13 and 14 such subunits respectively. The authors proposed that with one full rotation of the ATP synthase complex, three ATP are hydrolysed, and each c-subunit translocates one proton. As a consequence, the number of protons translocated per ATP is between 3.3 and 4.6 and with this, the smallest quantum of biologically conservable energy may range from 13 to 18 kJ per mol reaction.

In reversed electron transport as hypothesized for syntrophic butyrate and propionate oxidation, a high number of protons transported per ATP hydrolysed would allow ATP synthesis even at low energy gains. Hoehler and colleagues (2001) calculated minimal amounts of −10 to −19 kJ per translocated proton for organisms in anoxic methanogenic marine sediments. However, the number of membrane-integral c-subunits in ATP synthases of syntrophic bacteria has not been determined yet.

Genome analysis of *S. fumaroxidans* and *S. wolfei* indicates the presence of one kind of ATP synthase in each bacterium; the cytoplasmic F1 domain is encoded by Sfum_2581–2587 and Swol_2381–2385, and the membrane-integral F_0 domain is encoded by Sfum_1604–1605 and Swol_2387–2388 respectively. The ratio of transcription of F1 domain-coding genes to membrane-integral c-subunit-coding genes might give insight into the number of c-subunits per ATP synthase in *S. fumaroxidans* and *S. wolfei* in the future.

Buckel-Thauer Bcd/Etf

Most fermenting organisms have to regenerate their NAD^+ pool in the absence of external electron acceptors. It was assumed that in clostridia NADH is oxidized with ferredoxin, which in turn is oxidized with protons to form hydrogen. This reaction is endergonic and, until recently, it was not known how hydrogen-producing microorganisms could perform such a reaction while strongly accumulating hydrogen in their environment.

Clostridium kluyveri ferments ethanol plus acetate to a mix of butyrate, caproate and hydrogen. The critical step of NADH oxidation with ferredoxin was recently found to be catalysed by a butyryl-CoA dehydrogenase (Bcd)/electron-transferring flavoprotein subunit (Etf) complex which couples this endergonic reaction to the exergonic reduction of crotonyl-CoA to butyryl-CoA with NADH (Li *et al.*, 2008). Overall, two NADH molecules are oxidized and one molecule reduced ferredoxin (transferring two electrons) plus one molecule butyryl-CoA are formed (Li *et al.*, 2008) which we refer to as bifurcation. For butyrate-oxidizing bacteria, a reversal of this reaction was suggested, i.e. the endergonic reduction of NAD^+ with butyryl-CoA could be driven by the exergonic reduction of another NAD^+ with reduced ferredoxin (Herrmann *et al.*, 2008), a reaction that we refer to as confurcation. This mechanism could provide a concept for the reversed electron transport in syntrophic fatty acid degradation since homologues of this enzyme complex were found in genomes of the syntrophs *S. wolfei*, *S. fumaroxidans* and *P. thermopropionicum*, but not in *S. aciditrophicus*.

Confurcating/bifurcating [FeFe]-hydogenases

Apart from the Buckel-Thauer Bcd/Etf complex, another enzyme with bifurcating/confurcating activity was described recently, the [FeFe]-hydrogenase of *Thermotoga maritima* (Schut and Adams, 2009). *Thermotoga maritima* ferments glucose to acetate, CO_2 and H_2 via the Embden-Meyerhof pathway which generates two NADH and four reduced ferredoxins per molecule of glucose. In order to re-oxidize these carriers, the proposed confurcating [FeFe]-hydrogenase uses simultaneously electrons from NADH and reduced ferredoxin in a 1:2 ratio to produce hydrogen (Schut and Adams, 2009). This hydrogenase could not use either NADH or reduced ferredoxin alone for hydrogen production. Additionally, the authors found genes with sequence similarity to this trimeric [FeFe]-hydrogenase also in other organisms such as *S. fumaroxidans*, *P. thermopropionicum* and *S. wolfei* (Table 2, Fig. S1). Remarkably, our gene analysis indicated that some putative [NiFe]-hydrogenases and formate dehydrogenases in *S. fumaroxidans*, *P. thermopropionicum*, *S. wolfei* and *S. aciditrophicus* contain subunits with iron-sulfur-binding motifs and subunits homologous with the NADH dehydrogenase 51 kDa subunit, which is the NADH-binding subunit of Complex I. This indicates a possible confurcating function for [NiFe]-hydrogenases and formate dehydrogenases as well.

Rnf complex

In *Rhodobacter capsulatus* nitrogen fixation (*rnf*) genes were found which code for a membrane-bound enzyme complex that is most probably involved in energy transformation (Kumagai *et al.*, 1997). Gene analysis indicated that the encoded products RnfB and RnfC contain iron–sulfur clusters, RnfC contains potential NADH and FMN binding sites, and the membrane-bound RnfA, RnfD and RnfE are similar to subunits of the sodium-translocating NADH : quinone oxidoreductase (Kumagai *et al.*, 1997). The authors proposed that this complex translocates protons or sodium ions to drive the endergonic reduction of ferredoxin by NADH oxidation. Analogous *rnf* genes were found in numerous bacteria such as *Haemophilus influen-*

Table 2. General and genome-based characteristics of butyrate- and propionate-degrading syntrophic bacteria.

	Butyrate degraders		Propionate degraders	
Bacterial species	*Syntrophomonas wolfei* subsp. *wolfei*	*Syntrophus aciditrophicus*	*Syntrophobacter fumaroxidans*	*Pelotomaculum thermopropionicum*
Cell wall/morphology	Gram-positive rod	Gram-negative rod	Gram-negative rod	Gram-positive rod
Class	*Clostridia*	*Deltaproteobacteria*	*Deltaproteobacteria*	*Clostridia*
Pathway	β-Oxidation	β-Oxidation	Methyl malonyl CoA	Methyl malonyl CoA
Genome Genbank accession	CP000448	CP000252	CP000478	AP009389
Genes coding for confurcating FDHs and Hyds	Swol_1829-31 [Se]-FDH (FDH IV)	SYN_02138-40 [Se]-FDH	Sfum_2703-07 [Se]-FDH (FDH1)	PTH_2645-51 [Se]-FDH
	Swol_0783-86 [Se]-FDH (FDH II)	SYN_00629-35 [Se]-FDH	Sfum_0844-46 [FeFe]-Hyd (Hyd1)	PTH_1377-79 [FeFe]-Hyd
	Swol_1017-19 [FeFe]-Hyd	SYN_02219-22 [NiFe]-Hyd	Sfum_2713-16 [NiFe]-Hyd (Hyd4)	PTH_2010-12 [FeFe]-Hyd
		SYN_01369-70 [FeFe]-Hyd		
Genes coding for Tat-motif-containing FDHs			Sfum_3510-11 FDH (FDH3)	
and Hyds[a]	Swol_1823-26 [Se]-FDH (FDH III)	SYN_00602-05 [Se]-FDH	Sfum_0035-37 [Se]-FDH (FDH4)	PTH_1711-14 [Se]-FDH
		SYN_00632-35 [Se]-FDH	Sfum_1273-75 [Se]-FDH (FDH2)	PTH_1701-04 [NiFe]-Hyd
			Sfum_2952-53 [NiFe]-Hyd (Hyd2)	
Genes coding for other FDHs and Hyds			Sfum_3509 FDH	
			Sfum_0030-31 [Se]-FDH	
	Swol_0797-00 [Se]-FDH (FDH I)	Not present	Sfum_2220-22 [NiFe]-Hyd	Not present
			Sfum_3535-37 [NiFeSe]-Hyd	
			Sfum_3954-56 [NiFeSe]-Hyd	
Buckel-Thauer Bcd/Etf complex			Sfum_1371-73	PTH_0015-17
	Swol_0267-68	No complete complex	Sfum_3929-21	PTH_2000-02
			Sfum_3686-88	PTH_2431-33
Etf AB	Swol_0696-97	SYN_02636-37	Sfum_0106-07	PTH_1552-53
Rnf cluster	Not present	Syn_01658-64	Sfum_2694-99	Not present

a. Twin arginine translocation (Tat) motives indicate that the corresponding proteins are translocated through the cell membrane.
Gene locus tag numbers of genes in butyrate and propionate degraders, which show similarity with genes coding for energy-transforming protein complexes such as: formate dehydrogenases (FDH), hydrogenases (Hyd), electron-transferring flavoproteins (Etf), butyryl-CoA dehydrogenase (Bcd) and *Rhodobacter capsulatus* nitrogen fixation (Rnf) complexes.

zae, *E. coli*, *Acetobacterium woodii* and *Vibrio alginolyticus*, thus suggesting a general and important function for the Rnf complex in energy conservation (Nakayama *et al.*, 2000; Backiel *et al.*, 2008; Müller *et al.*, 2008).

Müller and colleagues (2008) investigated the function of an Rnf complex in the homoacetogenic bacterium *A. woodii*. Although biochemical proof has not been obtained yet, the authors found that caffeate respiration was coupled to ATP synthesis by a chemiosmotic mechanism with sodium ions as coupling ions, and that ferredoxin : NAD^+-oxidoreductase was the only membrane-bound enzyme detected in the pathway of H_2-dependent caffeate reduction. They postulated oxidation of ferredoxin with reduction of NAD^+ and the export of Na^+.

Methods used for gene analysis

Automatic annotations of genomes from DOE-Joined Genome Institute (IMG-JGI-DOE, version 2.9 August 2009, http://www.jgi.doe.gov/) were used to indicate the presence of gene clusters coding for formate dehydrogenases, hydrogenases, Buckel-Thauer Bcd/Etf complexes and Rnf clusters in *S. wolfei*, *S. aciditrophicus*, *P. thermopropionicum* and *S. fumaroxidans*. N-terminal amino acid sequences of FDH-1 and FDH-2 identified by de Bok and colleagues (2003) were used to find corresponding *fdh*-1 and *fdh*-2 nucleotide sequences in the genome of *S. fumaroxidans*. Pfam search (Sanger institute, 2009, http://pfam.sanger.ac.uk/search) was used to identify motifs in the amino acid sequences and the TMHMM Server v. 2.0 (Center for Biological Sequence Analysis, Technical University of Denmark, 2009, http://www.cbs.dtu.dk/services/TMHMM/) was used to identify transmembrane helices. With the TatP 1.0 Server twin-arginine translocation (Tat) motifs in the N-terminus were identified to predict protein localization in the cell (Bendtsen *et al.*, 2005). The incorporation of selenocystein (SeCys) was examined by RNA loop predictions with Mfold version 3.2 (Mathews *et al.*, 1999; Zuker, 2003). The RNA loop predicted in the 50–100 bp region downstream of the UGA-codon was compared with the consensus loop described by Zhang and Gladyshev (2005). Complete amino acid sequences of putative selenocystein-containing formate dehydrogenases were aligned to their homologues with CLUSTALX 1.81 (Kryukov and Gladyshev, 2004). SeCys incorporation was confirmed when the amino acid sequence aligned with conventional cystein in homologous proteins.

Topic 3. Hypotheses for energy conservation mechanisms in butyrate oxidizers and propionate oxidizers

Butyrate oxidation by S. wolfei

The electron transport in butyrate oxidation by *S. wolfei* was studied recently in a classical biochemical approach (Müller *et al.*, 2009). The electron transfer from butyryl-CoA to external electron acceptors was found to be inhibited by trifluoperazine, a compound known to inhibit electron transfer to menaquinone by the respiratory complex I in *Mycobacterium tuberculosis* (Yano *et al.*, 2006). Trifluoperazine also inhibited electron transfer from NADH to quinone analogues. An NADH-oxidizing enzyme complex was partially purified from the membrane fraction of *S. wolfei*. This activity was also found in the cytoplasmic fraction, especially after repeated treatment in the French Press cell, indicating that it is only superficially associated with the membrane (Müller *et al.*, 2009). The enzyme complex contained several proteins which were analysed by peptide mass fingerprinting and were compared via the known gene sequence with redox enzymes found in other bacteria, i.e. an enzyme system similar to the confurcating hydrogenase of *T. maritima* (Schut and Adams, 2009). Moreover, this hydrogenase homologue of *S. wolfei* appeared to be associated with homologues of an NADH-dependent formate dehydrogenase of *Eubacterium acidaminophilum* (Graentzdoerffer *et al.*, 2003), a bacterium that can also grow in syntrophic association with partner organisms (Zindel *et al.*, 1988). This enzyme complex could either act as a confurcating hydrogenase/formate dehydrogenase or as a proton-pumping NADH dehydrogenase (NDH) (Müller *et al.*, 2009). So far, it was not possible to show if this enzyme is directly linked to a transmembrane proton channel (Müller *et al.*, 2009). But even if the enzyme lacks such a channel protons could be transferred via a menaquinone cycle (Fig. 1) as described above for *E. coli* (Jormakka *et al.*, 2002). Whether the hydrogenase found really acts in a bifurcating manner as observed in *T. maritima* has still to be verified. So far, there is no indication of a ferredoxin-coupled redox reaction in butyrate oxidation by this bacterium.

Our results indicate that the 'Buckel-Thauer' reaction, i.e. a bifurcation of electrons from NADH with crotonyl-CoA and oxidized ferredoxin by the Bcd/EtfAB complex of *C. kluyveri* (Herrmann *et al.*, 2008; Li *et al.*, 2008), is not involved in butyrate oxidation by this bacterium. Until now, the function of *etf* genes in syntrophic butyrate degraders remains unclear. Possibly, the Bcd/EtfAB complex is expressed when *S. wolfei* grows by dismutation of crotonate. Whether other butyrate oxidizers, e.g. *S. aciditrophicus* or *S. buswellii*, employ the Bcd/EtfAB complex in butyrate oxidation remains an open question at this time.

Another interesting feature of the hydrogenase homologue of *S. wolfei* is its association with a formate dehydrogenase (Müller *et al.*, 2009). This supports older speculations that electrons from NADH oxidation are released as formate rather than hydrogen. The bacterium might even be able to choose which carrier it prefers, depending on the environmental conditions (Graentzdoerffer *et al.*, 2003), e.g. whether a partner is present which

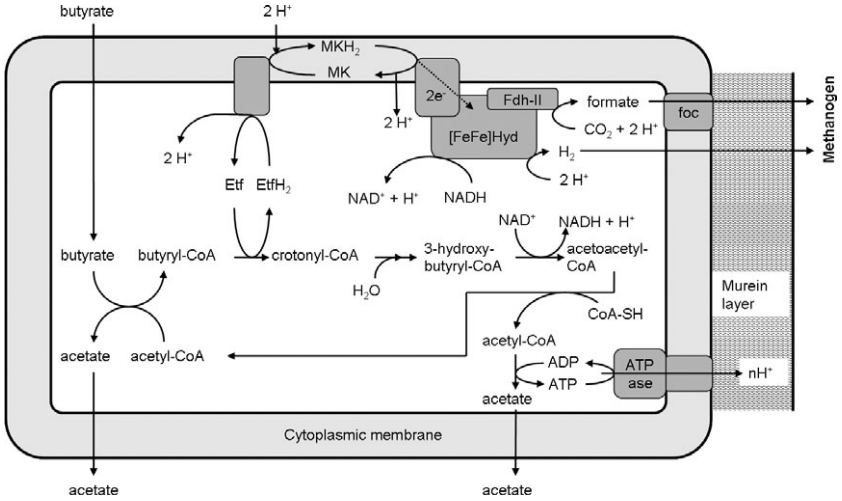

Fig. 1. Hypothetical energy-transforming mechanisms in the butyrate-degrading *Syntrophomonas wolfei*. The fatty acids acetate, butyrate and formate represent $acetate^- + H^+$, $butyrate^- + H^+$ and $formate^- + H^+$ respectively. (foc) represents a formate transporter.

consumes hydrogen, formate or both, and this preference might even differ between different butyrate oxidizers: co-culture experiments with *S. bryantii* and different partners (Dong *et al*., 1994) showed highest growth and substrate conversion rates with *Methanospirillum hungatei* which uses both hydrogen and formate, whereas co-cultures with the mainly formate-oxidizing *Methanobacterium formicicum* were slower, and there was no growth at all with the only hydrogen-consuming *Methanobrevibacter arboriphilus*. In contrast, it was shown earlier that *S. wolfei* grows in the presence of *M. arboriphilus*, although to a lower extent, indicating that *S. wolfei* can grow by interspecies hydrogen transfer only and formate plays only a minor role in electron transfer (Mcinerney *et al*., 1979; 1981).

Therefore, the electron transport during butyrate oxidation by *S. bryantii* might be different from that described above for *S. wolfei*, although the molecular prerequisites might be similar due to the close relatedness of both organisms. The formate dehydrogenase of *S. bryantii* was found to be membrane-bound and was most likely oriented to the periplasmic space whereas the partly membrane-bound hydrogenase was found in the cytoplasm and showed also activity with NAD^+ as electron acceptor (Dong and Stams, 1995). It was assumed that hydrogen is produced inside the cell while formate is produced outside. Additionally, an NADH dehydrogenase reacting with the tetrazolium dye MTT was measured, comparable to the described NADH:quinone oxidoreductase in *S. wolfei* which also reacts with MTT (Müller *et al*., 2009; N. Müller unpubl. data). It is tempting to speculate at this point that the described NADH dehydrogenase of *S. wolfei* could also be a bifurcating hydrogenase that couples NADH-dependent proton reduction with quinone reduction by another molecule of NADH. One electron pair would then be used to reduce protons to form hydrogen and the other one would be transferred to the external formate dehydrogenase. If hydrogenase and/or NADH dehydrogenase are coupled to the formate dehydrogenase via a quinone-mediated redox loop, two additional protons would be transferred outside the cell. Overall, proton consumption in the cytoplasmic space and proton release at the outside would result in a net proton gradient which in turn could drive menaquinol oxidation with NAD^+ or ADP phosphorylation by proton influx. This would require that formate and hydrogen both have to be kept at low concentration to allow the thermodynamically unfavourable reactions of CO_2 reduction with quinols and proton reduction with NADH. Although such a membrane-bound and quinone-oxidizing formate dehydrogenase has not yet been measured in *S. wolfei*, there are indications for such a system in its genome (Swol_0797–Swol_0799) (Table 2).

Although the formate/CO_2 couple and the hydrogen/proton couple are both at the same redox potential under physiological conditions, the question remains whether they are really equivalent inside the cell as assumed earlier (Schink, 1997). Co-cultures of *Moorella* sp. strain AMP and *Desulfovibrio* sp. strain G11 with formate as substrate in co-culture with hydrogen-only consuming methanogens converted formate to methane (Dolfing *et al*., 2008). It was assumed that formate is oxidized outside the cytoplasmic membrane, CO_2 and protons are released and electrons are shuttled to a membrane-bound hydrogenase facing the cytoplasm where protons are consumed (Dolfing *et al*., 2008). Thus, a net positive membrane potential could be formed without direct proton translocation but there is so far no proof that this reaction is coupled to energy conservation.

Propionate oxidation by S. fumaroxidans *and* P. thermopropionicum

The most difficult step in syntrophic propionate oxidation is the oxidation of succinate to fumarate. In the past,

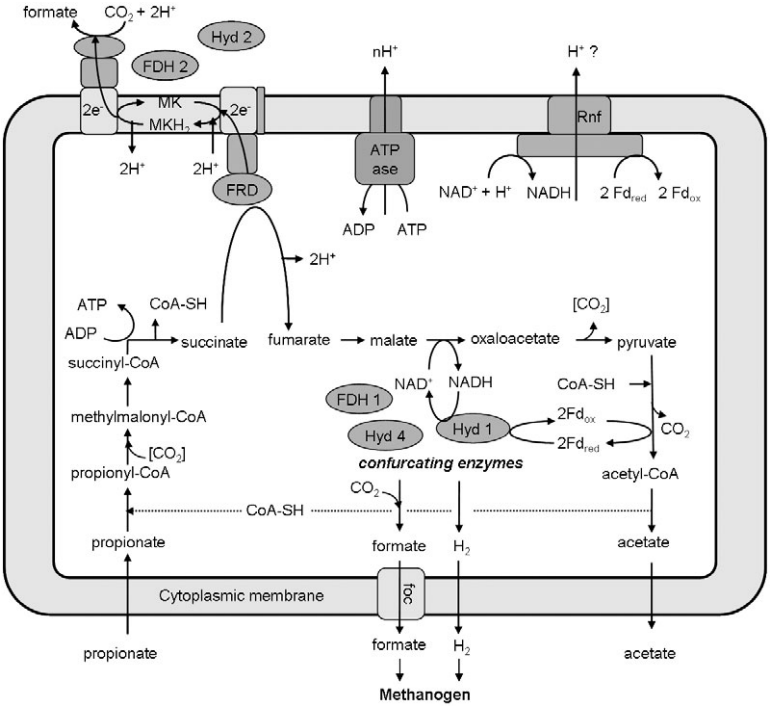

Fig. 2. Hypothetical energy-transforming mechanisms in the propionate-degrading *Syntrophobacter fumaroxidans*. The fatty acids acetate, propionate and formate represent acetate$^-$ + H$^+$, propionate$^-$ + H$^+$ and formate$^-$ + H$^+$ respectively. (foc) represents a formate transporter, [CO_2] a biotin-bound carboxylic group.

succinate dehydrogenases and fumarate reductases have been found to be similar based on their amino acid sequence (Lancaster, 2002). These authors classified fumarate reductases in five groups based on molecular composition. The fumarate reductase of *W. succinogenes* was classified within the group containing one hydrophobic subunit and two haem groups (Kröger *et al*., 2002). Our present gene analyses indicate that not only hydrophobic subunits of the fumarate reductase but also those of formate dehydrogenases of *W. succinogenes* (formate dehydrogenase delta subunits: WS0027, WS0736 and WS1148) contain haem groups and are homologous to cytochrome *b*.

Syntrophobacter fumaroxidans genome analysis revealed the presence of periplasmic formate dehydrogenases and hydrogenases (Fig. S1) as well as cytoplasmic fumarate reductases (Sfum_4092–4095, Sfum_1998–2000) which lack haem groups and a cytochrome *b*-like membrane-integrated domain. As such, fumarate reductases of *S. fumaroxidans* could not be classified within the five types described by Lancaster (2002). Instead, scattered over the genome, three cytochrome *b* (cytb561; Sfum_0091, cytb5; Sfum_3227 and cytb; Sfum_2932) and three cytochrome *c* homologous genes (Sfum_0090, Sfum_4047 and Sfum_1148) were found. Moreover, three genes with homology to cytochrome *b*:quinone oxidoreductases were found (Sfum_0339, Sfum_3009 and Sfum_3051). Cytochrome *b* and cytochrome *b*:quinone oxidoreductases possibly function in a similar way as the cytochrome-containing membrane-integrated domains of fumarate reductases, hydrogenases and formate dehydrogenases of *W. succinogenes* (Fig. 2). Candidates for periplasmic formate or hydrogen oxidation are formate dehydrogenase 2, 3 and 4 and hydrogenase 2 (Fig. 2, Fig. S1). These proteins may bind periplasmic cytochrome *c* and hydrophobic cytochrome *b* for succinate oxidation or fumarate reduction. Probably they also interconvert hydrogen and formate via a cytochrome *c* network, as proposed previously for the sulfate-reducing *Desulfovibrio vulgaris* Hildenborough (Heidelberg *et al*., 2004). Interconversion of hydrogen plus CO_2 and formate by *S. fumaroxidans* was observed by Dong and Stams (1995) and de Bok and colleagues (2002).

Formate dehydrogenase 1 of *S. fumaroxidans* was previously characterized (de Bok *et al*., 2003). It oxidizes formate with benzyl viologen as artificial electron acceptor, but NAD$^+$ did not support oxidation of formate. Based on the amino acid sequence, this selenocysteine-containing formate dehydrogenase is similar to FDHII of *S. wolfei* and to the NADH-dependent formate dehydrogenase of *Eubacterium acidaminophilum* (Graentzdoerffer *et al*., 2003). Whether formate dehydrogenase 1 can couple oxidation of both NADH and ferredoxin to CO_2 reduction in a manner analogous to proton reduction by the confurcating [FeFe]-hydrogenase of *T. maritima* was never tested. Based on our gene analysis, we hypothesize that hydrogenase 1, hydrogenase 4 and formate dehydrogenase 1 can couple the oxidation of NADH generated from malate oxidation with the oxidation of reduced ferredoxin generated from pyruvate oxidation to produce

hydrogen or formate (Table 2, Fig. 2). Especially transcription of genes coding for hydrogenase 1 (an [FeFe]-hydrogenase) appears to be upregulated when metabolic conversions generate NADH and reduced ferredoxin (P. Worm, A.J.M. Stams, X. Cheng and C.M. Plugge, unpublished). In *S. fumaroxidans* the Rnf complex might be used to conserve the energy of ferredoxin oxidation with NADH reduction by exporting protons, thus equilibrating the ratio of reduced ferredoxin to NADH to 1:1.

Compared with *S. fumaroxidans*, the genome of *P. thermopropionicum* contains less formate dehydrogenase- and hydrogenase-coding genes (Table 2). However, for each metabolic task, several candidates are present just as in *S. fumaroxidans*. In order to reoxidize the NADH and reduced ferredoxin that are generated during propionate degradation, *P. thermopropionicum* likely uses the confurcating formate dehydrogenase (PTH_2645–2649) and the two confurcating [FeFe]-hydrogenases (PTH_1377–1379 and PTH_2010–2012) (Kosaka *et al*., 2008). The produced formate would be transported through the membrane via a formate transporter (PTH_2651) of which the gene is located in the operon coding for the cytoplasmic formate dehydrogenase. The produced hydrogen diffuses through the membrane and is used by the methanogen.

Also similar to *S. fumaroxidans* is the mechanism of reversed electron transfer via fumarate reductase, a menaquinone loop and a periplasmic formate dehydrogenase (PTH_1711–1714) or hydrogenase (PTH_1701–1704) (Kosaka *et al*., 2008). Cytoplasmic and periplasmic formate dehydrogenases and hydrogenases could be used to interconvert formate and hydrogen. *Pelotomaculum thermopropionicum* can grow only with a hydrogen-using methanogen as its syntrophic partner (Ishii *et al*., 2005); however, formate is likely to generate hydrogen.

The high number of formate dehydrogenase- and hydrogenase-encoding genes in *S. fumaroxidans* likely provides *S. fumaroxidans* with more back-up possibilities when formate and hydrogen concentrations vary according to the activity of the partner methanogen. In contrast to *S. fumaroxidans*, *P. thermopropionicum* lacks an Rnf cluster and ferredoxin-reducing hydrogenases and formate dehydrogenases. *Syntrophobacter furmaroxidans* might use these mechanisms as alternatives to reoxidize NADH and ferredoxin, possibly with the use of an electron potential via the Rnf cluster, when environmental conditions change.

Concluding remarks

Recent biochemical studies and genome analyses indicated that *S. wolfei* uses electron-transferring flavoproteins coupled to a menaquinone loop to drive endergonic butyryl-CoA oxidation, and *S. fumaroxidans* uses a periplasmic formate dehydrogenase, cytochrome *b*:quinone oxidoreductases, a menaquione loop and a cytoplasmic fumarate reductase to drive endergonic succinate oxidation. Furthermore, we propose that confurcating [FeFe]-hydrogenases in *S. wolfei* and *S. fumaroxidans* are involved in NADH oxidation to form hydrogen. For both *S. wolfei* and *S. fumaroxidans*, a similar function is proposed for a formate dehydrogenase which would result in simultaneous hydrogen and formate transfer from the fermenting bacterium to the hydrogen- and formate-consuming syntrophic partner. *Syntrophobacter fumaroxidans* and *S. wolfei* are proposed to produce hydrogen and formate in the cytoplasm. *Pelotomaculum thermopropionicum* and *S. wolfei* are proposed to contain a mechanism to convert hydrogen into formate which would allow growth with hydrogen-only using methanogens. These proposed energy-converting mechanisms need biochemical verification. We hypothesize that they are key in syntrophic propionate- and butyrate-degrading communities, as well as in other syntrophic communities.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Schematic representation of putative formate dehydrogenases, hydrogenases and Rnf complexes in butyrate-degrading *Syntrophomonas wolfei* (A) and *Syntrophus aciditrophicus* (B), and in propionate-degrading *Syntrophobacter fumaroxidans* (C) and *Pelotomaculum thermopropionicum* (D). Gene locus tag numbers and α-, β- and γ-subunits are indicated in small characters, predicted iron–sulfur clusters and metal binding sites are indicated in capitals. 'Formate' represent $formate^- + H^+$.

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