

Interspecies electron transfer in methanogenic propionate degrading consortia

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Abstract

Propionate is a key intermediate in the conversion of complex organic matter under methanogenic conditions. Oxidation of this compound requires obligate syntrophic consortia of acetogenic proton- and bicarbonate reducing bacteria and methanogenic archaea. Although H₂ acts as an electron-carrier in these consortia, evidence accumulates that formate plays an even more important role. To make energy yield from propionate oxidation energetically feasible for the bacteria and archaea involved, the concentrations of H₂ and formate have to be extremely low. On the other hand, the diffusion distance of these carriers has to be small to allow high propionate conversion rates. Accordingly, the high conversion rates observed in methanogenic bioreactors are due to the fact that the propionate-oxidizing bacteria and their methanogenic partners form micro-colonies within the densely packed granules.

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1. Introduction

In methanogenic environments, organic compounds are degraded in the absence of light and inorganic electron acceptors such as oxygen, sulfate and nitrate. Under these conditions, the only possible microbial processes are fermentation and respiration with protons or bicarbonate as electron acceptors. Degradation of organic compounds requires at least three different trophic groups of microorganisms. Complex organic molecules are fermented by a variety of fermenting organisms to reduced organic compounds (e.g. lactate, ethanol, propionate and butyrate) and to compounds that can be used by methanogens directly (hydrogen, formate and acetate). Acetogenic bacteria oxidize the reduced organic products further to hydrogen, formate and acetate, and these are ultimately converted to methane and carbon dioxide by methanogens [1–3].

Anaerobic oxidation of the reduced organic compounds by acetogenic bacteria is thermodynamically unfavorable ($\Delta G^\circ > 0$), and is therefore only possible when the products are removed efficiently by the methanogens [4,5]. Propionate is one of the intermediates in the methanogenic degradation-process, which may account for a large fraction of the methane produced [6]. In this paper we review what is currently known about syntrophic propionate-oxidizing consortia, focussing on the syntrophic propionate-oxidizing bacteria, the thermodynamics of propionate oxidation, and interspecies electron transfer.

2. Thermodynamics of syntrophic propionate oxidation

Propionate is oxidized to acetate, bicarbonate and hydrogen or formate (reactions 1 and 2, Table 1). If this reaction is coupled to methanogenesis (reactions 3–6, Table 1), it can be calculated that the maximum amount of energy released is equivalent to about 1 ATP, assuming that the minimum amount of energy required

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Table 1
Reactions involved in syntrophic oxidation of propionate in methanogenic ecosystems

	Reaction equation			ΔG° (kJ/mol)	
				25°C	55°C
1	Propionate ⁻ + 3H ₂ O	→	Acetate ⁻ + HCO ₃ ⁻ + H ⁺ + 3H ₂	+76.1	+62.3
2	Propionate ⁻ + 2HCO ₃ ⁻	→	Acetate ⁻ + H ⁺ + 3HCOO ⁻	+72.2	+59.7
3	H ₂ + $\frac{1}{4}$ HCO ₃ ⁻ + $\frac{1}{4}$ H ⁺	→	$\frac{1}{4}$ CH ₄ + $\frac{3}{4}$ H ₂ O	-33.9	-30.6
4	Formate ⁻ + $\frac{1}{4}$ H ₂ O + $\frac{1}{4}$ H ⁺	→	$\frac{1}{4}$ CH ₄ + $\frac{3}{4}$ HCO ₃ ⁻	-32.6	-29.7
5	Acetate ⁻ + H ₂ O	→	CH ₄ + HCO ₃ ⁻	-31.0	-34.7
6	Propionate ⁻ + H ₂ O	→	$1\frac{3}{4}$ CH ₄ + $1\frac{1}{4}$ HCO ₃ ⁻ + $\frac{1}{4}$ H ⁺	-56.4	-65.0

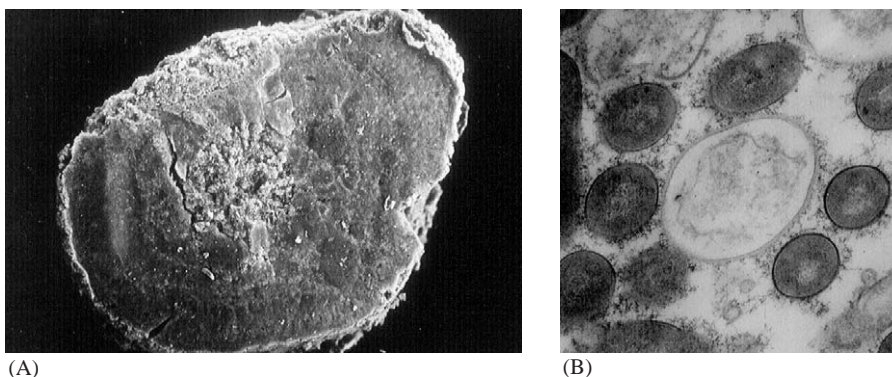


Fig. 1. (A) Scanning electron micrograph of a granule from an upflow anaerobic sludge bed reactor (UASB). (B) Transmission electron micrograph of an ultrathin section of a granule from a UASB-reactor, showing a syntrophic acetogen in the middle, surrounded by methanogenic archaea. The images were kindly provided by Tim Grotenhuis.

to synthesize 1 mol of ATP, is 60 kJ/mol [4]. Since there are no methanogens that are both acetoclastic and hydrogenotrophic (these latter often use both H₂ and formate), this amount of energy has to be shared by three different species. This suggests that each species involved yields only 1/3 of an ATP unit per propionate molecule converted to CO₂ and CH₄. It was also calculated for the conditions, which normally prevail in anoxic bioreactors, that each of the microorganisms involved (in propionate oxidation) has about -20 kJ/mol available [7]. Assuming that three protons are translocated across the charged cytoplasmic membrane, this amount is the equivalent of one proton translocated across the charged cytoplasmic membrane [4,8]. However, since it is possible that the H⁺/ATP coupling ratio is 4 rather than 3 [9], the amount of energy available per proton translocated may be even less than 20 kJ/mol. For syntrophic propionate oxidation it was reasoned that the bacteria have to spent 2/3 of the ATP, which is released by substrate-level phosphorylation, to translocate protons to the periplasmic space in order to drive the energetically unfavorable oxidation of succinate to fumarate [4,10]. This phenomenon is known as ‘reversed

electron transport’, but unfortunately evidence for its occurrence has only been presented for syntrophic butyrate- and glycolate-oxidizing bacteria so far [4].

Since syntrophic degradation so strongly depends on removal of the products, the diffusion distance between the producing and the consuming microorganism may have a large effect on the rate of propionate conversion [11]. In granular sludge containing bioreactors the extremely high cell densities and the short interbacterial distances may therefore explain the high rates of methane formation (Fig. 1). This will be discussed in more detail in the section about interspecies hydrogen and formate transfer.

Temperature may also affect the thermodynamics of syntrophic propionate-oxidation. Using the van ‘t Hoff equation it can be calculated that hydrogen formation becomes energetically more favorable at higher temperatures, whereas hydrogen consumption by the methanogens becomes less favorable (Table 1). However, since diffusion coefficients become higher and diffusion gradients steeper, it can be expected that propionate conversion rates are higher at elevated temperatures.

3. Syntrophic propionate-degrading bacteria

The first syntrophic propionate-oxidizing bacterium, *Syntrophobacter wolinii*, was enriched from an anaerobic sewage digester [12]. The organism was obtained in co-culture with a *Desulfovibrio* sp., which reduced sulfate in this culture with the reducing equivalents released from the oxidation of propionate. A methanogenic culture with *Methanospirillum hungatei* was obtained as well, but unfortunately attempts to remove the sulfate reducer from this culture failed. Other syntrophic propionate-oxidizing bacteria have been described since then, but none of these was obtained in pure or defined co-culture until 1993, when phylogenetic analysis revealed a relationship of *S. wolinii* to sulfate-reducing bacteria [13]. Soon thereafter, *Syntrophobacter wolinii* was obtained in pure culture on propionate plus sulfate, while pyruvate also appeared to support axenic growth [14]. Several other syntrophic propionate-oxidizing bacteria have been isolated, including two other *Syntrophobacter* species. The *Syntrophobacter* strains form a cluster within the δ -subclass of the proteobacteria [15] (Fig. 2). All of these strains oxidize propionate to acetate, CO₂ and H₂ or formate, while they differ from each other with respect to morphology and additional substrates used. Propionate is oxidized via the methyl-malonyl-CoA pathway, which is basically the reversal of the fermentative pathway of propionate formation [16]. *S. fumaroxidans* was obtained in pure culture on fumarate, which is an intermediate of this pathway

[17]. *S. wolinii* also appeared to be able to ferment this compound [18]. In addition, *S. fumaroxidans* is able to grow by fumarate reduction coupled to the oxidation of hydrogen, formate or propionate [10,19]. Without external electron donors, the organism disproportionates fumarate to CO₂ and succinate. *S. wolinii* is not able to oxidize fumarate further than acetate, while *S. pfennigii* does not catabolize fumarate at all [20]. A markedly different organism is *Smithella propionica*, which was isolated by Liu and co-workers, [18]. This organism produces much less methane from propionate than the *Syntrophobacter* strains, and besides acetate it produces small amounts of butyrate. The pathway of propionate conversion in this organism was investigated by ¹³C-NMR [21]. It was suggested that the first step is condensation of propionate to a six-carbon intermediate, which, after some rearrangements, is ultimately cleaved into butyrate and acetate. With this pathway the results of previous NMR-studies could also be explained, since it was found that in sludge and enrichment cultures the randomizing methyl-malonyl-CoA pathway could not account for the labeling patterns observed when either 2- or 3-¹³C-propionate was added. While all of the organisms described above are mesophilic organisms, thermophilic propionate-oxidizing bacteria have also been described, and two of these have been obtained in pure culture so far: *Pelotomaculum thermopropionicum* strain SI, and *Desulfotomaculum thermobenzoicum*, subsp. *thermosyntrophicum* [22–24]. *Desulfotomaculum thermocicternum* was also described

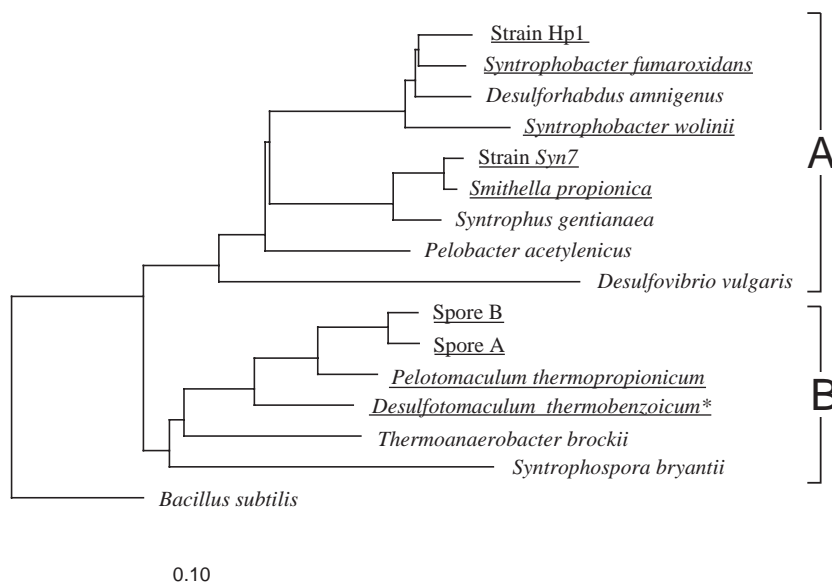


Fig. 2. Neighbor joining tree based on 16S rDNA sequences, showing the phylogenetic position of syntrophic propionate-oxidizing bacteria among other syntrophic bacteria. The bar represents 10% estimated sequence divergence. **Desulfotomaculum thermobenzoicum* subspecies *thermosyntrophicum*: (A) δ -subdivision of the proteobacteria and (B) low G+C gram positives. Syntrophic propionate degrading bacteria are underlined.

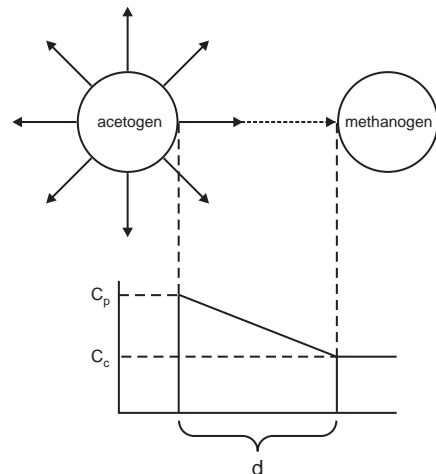
to oxidize propionate syntrophically, but the authors, who described the other two thermophilic syntrophs, could not reproduce this [22,24,25]. Unlike the mesophilic propionate-oxidizers discussed here, these organisms are gram-positive spore-forming bacteria and are phylogenetically related to *Desulfotomaculum* species (Fig. 2). *Pelotomaculum thermopropionicum* Strain SI is closely related to two mesophilic spore-forming propionate-oxidizing bacteria, ‘Spore A’ and ‘Spore B’ which were enriched previously in our laboratory [26]. A microorganism with similar physiological properties was one of the dominant acetogens in granules of an UASB-reactor fed with a mixture of acetate, propionate and butyrate [27]. Other highly purified enrichments of syntrophic propionate-oxidizing bacteria described so far are strain SYN7 and strain HP1.1. SYN7 is related to syntrophic benzoate-oxidizing strains of the genus *Syntrophus* [26], while strain HP 1.1 is more related to members of the *Syntrophobacter* genus [28] (Fig. 2).

4. Interspecies hydrogen and formate transfer

Syntrophic interactions are usually associated with interspecies H₂ transfer, although formate transfer has also been considered as a possible mechanism. Possibly even other mechanisms exist as well. The H₂ and formate concentrations in syntrophic cultures are extremely low, and therefore it is difficult to determine which compound is the most important electron carrier. Furthermore, many of the syntrophs involved are able to produce both hydrogen and formate, while most of the methanogenic or sulfidogenic partners are able to oxidize both compounds. In addition, organisms, which metabolize both compounds, are usually also able to interconvert H₂/CO₂ and formate [7]. Schmidt and Ahring [29] studied the effects of granule-disintegration and they concluded that formate could not account for the decrease in the propionate and butyrate degradation rates in their studies. In contrast, formate transfer was the most important mechanism in flocs from a reactor treating whey [30]. It has even been calculated that for suspended propionate- and butyrate-degrading cultures [31] formate transfer was even about 100 times more important than H₂-transfer.

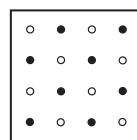
Important parameters in such studies are the solubility and the diffusion constants of hydrogen and formate. Since the solubility of hydrogen is much less than the solubility of formate, a much higher concentration difference between producing and consuming organisms is possible; this concentration difference can be up to a 1000 times higher with formate than with hydrogen. Hydrogen however, diffuses approximately 30 times faster than formate in water. The flux of compounds transferred between the organisms involved depends on the surface area of the producing bacterium

(A), the diffusion constant (*D*), the concentration gradient (*C_p*–*C_c*) and the distance between the organisms (*d*) (Fig. 3A). Therefore it can be expected from this equation, that formate transfer is the favored mechanism when the interbacterial distances are high, while hydrogen transfer becomes more favorable when these distances are small. To illustrate this, the flux was calculated for a situation in which the cells are equally distributed and compared to a scenario in which they are clustered (Fig. 3B). Since both mechanisms for electron transfer may occur at the same time, the efficiency



(A) Flux = $A \cdot D \cdot (C_p - C_c) / d$ mol sec⁻¹

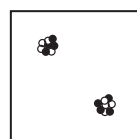
equal distribution



$r = 1 \text{ }\mu\text{m}$
 $d = 8 \text{ }\mu\text{m}$
 $C_p = 0.05 \text{ }\mu\text{M}$
 $C_c = 0.005 \text{ }\mu\text{M}$

Flux = 10 nmol ml⁻¹ min⁻¹

cluster formation



$r = 1 \text{ }\mu\text{m}$
 $d = 0.08 \text{ }\mu\text{m}$
 $C_p = 0.05 \text{ }\mu\text{M}$
 $C_c = 0.005 \text{ }\mu\text{M}$

Flux = 1000 nmol ml⁻¹ min⁻¹

(B)

Fig. 3. Effect of interbacterial distance on the flux of interspecies electron carriers between a producing (acetogen) and a consuming (methanogen) organism: (A) calculation of the flux between the producing and consuming organism and (B) effect of diffusion distance on the flux between the two types of bacteria either in suspended or aggregated distribution. In this example, the flux was calculated for H₂ with 10⁹ cells with an average diameter of 2 μm (adapted from Schink and Tauer [11]). *C_p*, concentration at the surface of the producing bacterium; *C_c*, concentration at the surface of the consuming bacterium; *r*, radius of bacteria; *d*, average distance between organisms.

probably increases with increasing cell density. Accordingly, it was found that shortening of the interbacterial distances by creating artificial precipitates or addition of methanogens led to increased propionate-degradation rates [29,32].

The involvement of hydrogen and formate as electron carriers during syntrophic growth was thoroughly investigated for the propionate-oxidizing bacterium *Syntrophobacter fumaroxidans*. This organism could not be cultured with *Methanobrevibacter arboriphilicus*, a strain, which only uses H₂ and not formate [33]. However, this could also be due to the fact that the threshold for H₂ for this particular methanogen (*M. arboriphilicus*) is too high [34]. Later, Dong and Stams [35] calculated that only a formate-flux could account for the methane production rates that were measured with suspended co-cultures of *S. fumaroxidans* and *M. hungatei*. The parameters they used for their calculations and the corresponding fluxes are presented in Table 2, assuming an equal distribution of the cells as depicted in Fig. 3B.

Further studies focused on the biochemical aspects of hydrogen and formate transfer. Analysis of cells of *S. fumaroxidans* which were grown axenically on fumarate, revealed that the organism possesses both hydrogenase as well as formate dehydrogenase activity [10]. It was also demonstrated that *S. fumaroxidans* cells produce both hydrogen and formate when grown axenically on propionate plus fumarate [35]. However, hydrogenases are known to have a central role in the metabolism of many anaerobic bacteria, while a formate dehydrogenase was already expected to function in the metabolism

of this organism, since it is very likely that the organism fixes carbon dioxide via the reversal of the acetyl-CoA cleavage pathway during syntrophic growth [36]. Recently, the hydrogenase and formate dehydrogenase levels were studied in pure cultures of *S. fumaroxidans* as well as in co-cultures (with *M. hungatei*). Co-cultured cells were separated (quantitatively) by percoll gradient centrifugation). These studies revealed that both the hydrogenase and the formate dehydrogenase levels are highest in syntrophically cultured cells. Furthermore it was demonstrated that the organism possesses at least two formate dehydrogenases and three hydrogenases [37–39]. Two formate dehydrogenases and one of the hydrogenases were purified from *S. fumaroxidans*. Both formate dehydrogenases exhibited extremely high formate oxidation and CO₂-reduction rates as compared to enzymes purified from other anaerobic bacteria and archaea. Based on their localization in the cell, it was hypothesized that one of these enzymes is involved in the pathway of CO₂-fixation (reversed acetyl-CoA pathway), while the other enzyme is used to reduce CO₂ (to formate) at the periplasmic side of the cytoplasm [39]. The role of each of the individual hydrogenases is not yet understood [40]. Altogether, these data provided strong evidence that formate plays an important role at least in suspended syntrophic propionate-degrading co-cultures.

Although acetate is also an intermediate of (methanogenic) propionate degradation, extremely low concentrations of this compound are not absolutely necessary for syntrophic growth. Since only one mol of acetate is produced per mol propionate oxidized, against three moles of hydrogen or formate (Table 1), the advantageous effect of acetate removal on the propionate oxidation rate is much less than that for hydrogen or formate. However, addition of acetate may inhibit propionate- and butyrate oxidation, while removal of acetate is known to stimulate growth on these compounds [41–44]. It can easily be calculated that the $\Delta G'$ of propionate oxidation increases by increasing the acetate concentration [7]. Removal of acetate may therefore allow a steeper gradient of hydrogen and formate between acetogen and methanogen, which could result in an increased flux.

Table 2

Calculated H₂ and formate flux in suspended co-cultures of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*, according to Dong and Stams [35]

Cell size of acetogen	1.1–1.6 × 1.8–2.5 μm
Cell numbers in co-culture at the end of growth	9.6 × 10 ⁸
Total cell surface area of <i>S. fumaroxidans</i> cells	1.8 × 10 ⁹ μm ²
Average distance between cells	12.4 μm
Measured CH ₄ formation of co-culture	0.42 nmol ml ⁻¹ min ⁻¹
Highest H ₂ level formed by <i>S. fumaroxidans</i>	6.8 Pa
Highest formate level formed by <i>S. fumaroxidans</i>	24 μM
Lowest H ₂ level reached by <i>M. hungatei</i>	2 Pa
Lowest formate level reached by <i>M. hungatei</i>	15 μM
H ₂ flux	1.13 nmol ml ⁻¹ min ⁻¹
Formate flux	117 nmol ml ⁻¹ min ⁻¹

5. Syntrophic interactions in aggregated biomass

Propionate-oxidizing *Syntrophobacter*-like bacteria have been identified in micro-colonies in intimate association with methanogens [45]. For kinetic reasons the spatial organization of the microorganisms in aggregated biomass is critical, as mentioned above. Because of the need for such close proximity, random cell-cell associations would lower the metabolic efficiency. Organization at a larger scale in sludge granules

is observed in the distribution of distinct species [46,47] and of distinct metabolic processes [48]. The microbial composition of sludge granules originating from an UASB reactor treating potato-processing factory wastewater changed when they were incubated in a lab scale UASB reactor with propionate as the sole substrate. Syntrophic propionate-oxidizing bacteria developed rapidly inside the sludge granule in defined microcolonies with methanogenic archaea. Moreover, the methanogenic partner in the sludge granules, *Methanospirillum*-like cells, was intertwined with all the microcolonies in the granules. This is in contrast with *Methanobrevibacter*-like methanogens that are usually juxtapositioned to the hydrogen-producing propionate oxidizers.

The mechanisms underlying the process of granulation are at this point still poorly understood, but perhaps investigating cell-to-cell signaling mechanisms will provide an outcome to this problem [49]. During our experiments performed with syntrophically propionate-degrading co-cultures of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*, cells were always present in suspension. However, we do have some indications that one of these organisms is able to trigger aggregation. What actually causes aggregation of these co-cultures is still unclear, but preliminary experiments showed that at least bacterial density and ionic strength are important key factors. Perhaps propionate-degrading consortia play a key role in the granulation process, and further research on this topic should therefore preferably focus on cell-to-cell signaling mechanisms.

6. Conclusions

- Under methanogenic conditions propionate oxidation requires obligate syntrophic consortia of acetogenic and proton- and bicarbonate reducing bacteria and methanogenic archaea.
- The amount of energy released from the complete oxidation of propionate (under methanogenic conditions) is 1 ATP, which has to be shared by three different organisms.
- The majority of the propionate-oxidizing bacteria oxidize propionate via the methyl-malonyl-CoA pathway yielding acetate, CO₂ and H₂ or formate. However, propionate can also be oxidized via a second pathway. In this novel pathway propionate is condensed to a six-carbon intermediate, which is ultimately cleaved to butyrate and acetate.
- Hydrogen is an important interspecies electron carrier, but evidence accumulates that for syntrophic propionate oxidation formate may be even more important.
- The high conversion rates in aggregated biomass are largely due to the small interbacterial distances,

allowing more efficient transfer of the interspecies electron carriers hydrogen and formate.

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