# Role of Formate and Hydrogen
# in the Syntrophic Degradation of
# Propionate and Butyrate

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# Role of Formate and Hydrogen in the Syntrophic Degradation of Propionate and Butyrate

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# PROPOSITIONS

1. During the period when interest in the pathways of intermediary metabolism was at its height, anaerobic bacteria were star performers in the repertoire of the microbiologist. -- Thauer & Morris, 1984.

2. The letter "R" is the most difficult one to pronounce in many kinds of languages, especially the Dutch "R" for chinese people.

3. It is indeed necessary for a "sandwich" Ph.D. student to have the two thick outer layers to get enough "nutrition".

4. Interspecies $H_2$ or formate transfer: that is the question.

5. Syntrophism exists not only in the microbial world, but also in the human society.

6. Music calms you when you are excited, encourages you when you are depressed, and consoles you when you are distressed.

7. When you ask a question, Chinese will deny the question itself, while English will deny the question of its meaning.

8. The importance of syntrophic degradation is well established for compounds which can not be degraded in another way. However, the importance of syntrophic consortia

in the degradation of other compounds is often underestimated.-- Stams, 1994.

9. One can reduce the size of manuscript by using the compact language Chinese.

10. When one is only eager to reach the final achievement, one will miss the joy of failures and success.

11. A good scientist is the one who can demonstrate a significant principle with a simple approach.

12. A frog at the bottom of a well only sees a piece of the sky.

# Contents

## Chapter 1

# General introduction

## Mineralization of organic complex in methanogenic environments

In methanogenic environments mineralization of complex organic matter to the most oxidized form of carbon ($CO_2$) and the most reduced form of carbon ($CH_4$) is carried out sequentially. Ultimately, $CO_2$ is the terminal sink of electrons in a series of oxidative reactions. Methanogenic environments are widely distributed in nature. These include, wetwoods of living trees (Schink et al. 1981) where cellulose and pectins are degraded anaerobically, digestive tracts of higher animals and insects (Hungate 1966; Bryant 1977; Wolin 1981), and freshwater sediments (Rudd & Taylor 1980; Crill et al. 1991). Anaerobic bioreactors are artificial methanogenic environments, which are used for e.g. the stabilization of sewage sludge and for the treatment of a variety of industrial wastes. The widest applied bioreactor for anaerobic wastewater treatment is the UASB (Upflow Anaerobic Sludge Bed) reactor (Lettinga & Hulshoff Pol 1991) which contains well settleable methanogenic granular sludge with a high methanogenic activity. Other types of anaerobic bioreactors are the Fluidized Bed reactor (Iza 1991), the Anaerobic Filter (Young 1991) and the Expanded Granular Sludge Bed reactor (de Man et al. 1988).

Different physiological types of bacteria are involved in the methanogenic mineralization of complex organics. Methanogens occupy the terminal position of this anaerobic food web. They are specialized in the degradation of a very limited number of substrates. *Methanobacterium bryantii* (Bryant et al. 1967) and *Methanobrevibacter arboriphilus* (Zeikus & Henning 1975; Zehnder & Wuhrmann 1977) utilize only $H_2/CO_2$, *Methanothrix* (*Methanosaeta*) sp. (Huser et al. 1982; Patel & Sprott 1990) only use acetate while e.g.

*Methanospirillum hungatei* and *Methanobacterium formicicum* can use $H_2/CO_2$ and formate (Schauer et al. 1982; Bryant & Boone 1987). The most versatile methanogens are *Methanosarcina* sp.; they can use $H_2/CO_2$, acetate, methanol, methylated amines and pyruvate (Whitman et al. 1992; Jones 1991). During methanogenesis, organic polymers are first hydrolyzed and fermented by fermentative organisms to the methanogenic precursors $H_2/CO_2$, formate, and acetate, and to reduced organic compounds, such as propionate, butyrate, lactate, succinate and ethanol. These reduced compounds can be oxidized further to the methanogenic substrates by acetogenic bacteria (Mah et al. 1990; Stams 1994). Figure 1 shows how in methanogenic environments the degradation of organic matter is achieved by consortia of fermentative organisms, acetogenic and methanogenic bacteria, about 76% of organic matter is degraded via reduced organic intermediates (Toerien & Hattingh 1969; Wolin 1974; Zehnder 1978; Bryant 1979). Therefore, acetogenesis is a key process in the mineralization of organic matter in methanogenic environments.

## Syntrophic degradation

Syntrophism is defined as that the cooperation of partners depends entirely on each other to perform the metabolism (Schink 1992). Under methanogenic conditions, almost all the acetogenic reactions can only be carried out by obligate syntrophic consortia (Schink 1992; Stams 1994). This as a result of the fact that the oxidation of these compounds is energetically unfavorable (table 1), and/or that at least one intermediate step involved in

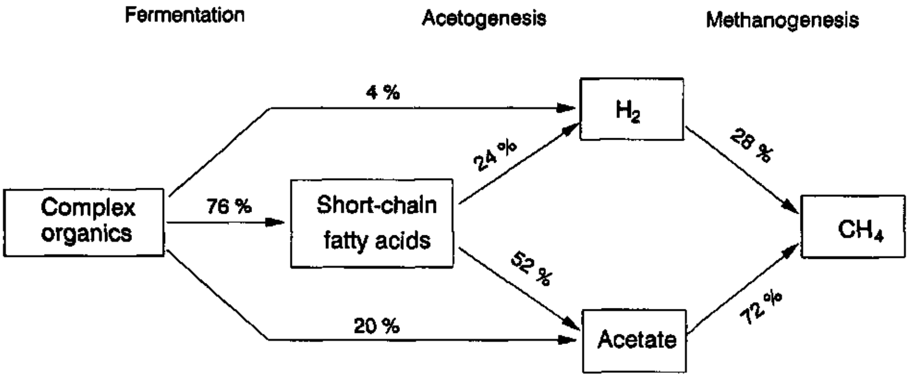

Fig. 1. Methanogenic mineralization of complex organic matter and the percentage flow of the energy content involved in complex matter to methane formation (From McCarty, 1982).

their degradation is energetically difficult. The products $H_2$, formate and to some extent also acetate have to be removed by methanogens to "pull" the reactions. Low concentrations of $H_2$ and formate, and in some cases also low acetate concentration are essential. The highest $H_2$, formate and acetate concentrations that can be tolerated during syntrophic oxidation are dependent on the thermodynamics of the oxidative reactions (table 1). E.g. the maximum $H_2$ partial pressure that can be tolerated is 14.5, 45 and 1000 Pa for propionate, butyrate and ethanol oxidation, respectively. For these calculations, it was assumed that $CH_4$ partial pressure is at 2500 Pa, the concentrations of acetate, propionate, butyrate and bicarbonate are 25 mM. Methanogens catabolize $H_2$, formate and acetate efficiently; the reported threshold values for $H_2$ are around 10 Pa (59 nM in liquid), and those for formate and acetate are around 10 $\mu$M, respectively (Cord-Ruwisch et al. 1988; Schauer et al. 1982; Jetten et al. 1990).

Table 1. Oxidative reactions completed by syntrophic consortia with proton as electron acceptor

| Reactions | $\Delta G^{\circ\prime}$(kJ/mol) | Organisms in consortia | Reference |
|---|---|---|---|
| Acetate$^-$ + $4H_2O$ ---> $2HCO_3^-$ + $4H_2$ + $H^+$ | +104.6 | "Reversibacterium" + *Methanobacterium* strain THF | 1 |
| Propionate$^-$ + $3H_2O$ ---> acetate$^-$ + $HCO_3^-$ + $3H_2$ + $H^+$ | +76.1 | *Syntrophobacter wolinii* + *Desulfovibrio* | 2 |
| | | *Syntrophobacter wolinii* + *Methanospirillum* sp. | 3 |
| | | MPOB + *Methanospirillum* sp. | 4 |
| Butyrate$^-$ + $2H_2O$ ---> 2acetate$^-$ + $2H_2$ + $H^+$ | +48.1 | *Syntrophomonas wolfei* + *Methanobacterium bryantii* | 5, 6 |
| | | *S. sapovorans* + *M. hungatei* | 7 |
| | | *Syntrophospora bryantii* + *M. hungatei* | 8 |
| | | Unamed + *Methanobacterium thermoautotrophicum* | 9 |
| Caproate$^-$ + $3H_2O$ ---> 3acetate$^-$ + $4H_2$ + $2H^+$ | +96.2 | Strain IB + *Methanobacterium formicicum*<br>+ *Methanosarcina mazai* | 10 |
| Isovalerate$^-$ + $HCO_3^-$ + $H_2O$ ---> 3acetate$^-$ + $H_2$ + $H^+$ | +20.2 | Strain Gralval and Gölval + *M. hungatei* | 11 |
| Methanol + $2H_2O$ ---> $HCO_3^-$ + $2H_2$ + $H^+$ | +23.5 | *Sporomusa acidovorans* + *M. hungatei* | 12 |
| Ethanol + $H_2O$ ---> acetate$^-$ + $2H_2$ + $H^+$ | +9.6 | S-organism + *M. bryantii* | 13 |
| | | *Desulfovibrio* + $SO_4^=$ | 14 |
| | | *Pelobacter venetianus* + *M. hungatei*<br>or+ *Acetobacterium woodii* | 15 |
| | | *P. carbinolicus* + *M. hungatei*<br>or + *Acetobacterium woodii* | 16 |
| | | *P. acetylenicus* + *M. hungatei* | 17 |
| Lactate$^-$ + $2H_2O$ ---> acetate$^-$ + $HCO_3^-$+ $3H_2$ + $H^+$ | -4.2 | *Desulfovibrio* sp. + *Methanosarcina barkeri* | 18 |
| Benzoate$^-$ + $7H_2O$ ---> 3acetate$^-$ + $HCO_3^-$+ $3H_2$ + $3H^+$ | +58.9 | *Syntrophus buswellii* + *M. hungatei*<br>or + *Desulfovibrio* sp. | 19 |
| Phenol + $5H_2O$ ---> 3acetate$^-$ + $2H_2$ + $3H^+$ | +5.5 | Strain P-2 + *Wolinella succinogenes* | 20 |

| | | | |
|---|---|---|---|
| Glycolate$^-$ ---> glyoxylate$^-$ + $H_2$ | +38 | Strain FlGlyR + *M. hungatei* | 21, 22 |
| Alanine + $3H_2O$ ---> acetate$^-$ + $HCO_3^-$ + $2H_2$ + $H^+$ + $NH_4^+$ | +7.5 | *Acidaminobacter hydrogenoformans* + *Desulfovibrio* HL21 | 23 |

Reference: 1. Zinder and Koch 1984; 2. Boone and Bryant 1980; 3. Dörner 1992; 4. Stams et al. 1993; 5. McInerney et al. 1979; 6. McInerney et al. 1981; 7. Roy et al. 1986; 8. Stieb and Schink 1985; 9. Henson and Smith 1985; 10. Wu et al. 1994; 11. Stieb and Schink 1986; 12. Cord-Ruwisch and Ollivier 1986; 13. Bryant et al. 1967; 14. Bryant et al. 1977; 15. Schink and Stieb 1983; 16. Schink 1984; 17. Schink 1985; 18. McInerney and Bryant 1981; 19. Mountfort and Bryant 1982; 20. Barik et al. 1985; 21. Friedrich et al. 1991; 22. Friedrich and Schink 1993; 23. Stams and Hansen 1984.

## Electron transfer in syntrophic consortia

In obligate syntrophic processes, reducing equivalents are disposed of by reduction of protons or bicarbonate leading to the formation of $H_2$ and formate, respectively. Common electron mediators in acetogenic bacteria are NAD, e.g. involved in the dehydrogenation of malate (Plugge et al. 1993; Houwen et al. 1990) and of 3-hydroxy-butyryl-CoA (Schink 1992), FAD, involved in e.g. the oxidation of butyryl-CoA (Schink 1992) and of succinate, and ferredoxin, possibly involved in the oxidative decarboxylation of pyruvate. However, the redox potentials of these electron mediators are higher than those of $H^+/H_2$ or $HCO_3^-/HCOO^-$ (table 2). So, transfer of electrons from reduced electron mediators to either proton or bicarbonate is energetically unfavorable, unless the $H_2$ partial pressure or the formate level are kept extremely low. This can be illustrated by taking NADH oxidation coupled to proton reduction and to bicarbonate reduction as example:

| | $\Delta G^{\circ\prime}$ (kJ/mol) |
|---|---|
| $NADH + H^+ \longrightarrow NAD^+ + H_2$ | **+18.1 (1)** |
| $NADH + HCO_3^- + H^+ \longrightarrow NAD^+ + HCOO^- + H_2O$ | **+16.8 (2)** |

The Gibb's free energy change ($\Delta G'$) can be calculated according to the following equations:

$$\Delta G' = \Delta G^{\circ\prime} + RTln\frac{[NAD^+]\cdot P_{H_2}}{[NADH]\cdot[H^+]} \qquad \Delta G' = \Delta G^{\circ\prime} + RTln\frac{[NAD^+]\cdot[HCOO^-]}{[NADH]\cdot[H^+]\cdot[HCO_3^-]}$$

Where $\Delta G^{\circ\prime}$ represents free energy change at standard conditons, T=298°K, pH 7, 1 M for solutes, and $10^5$ Pa for gases, and R is the gas constant (8.24 $kJ.mol^{-1}.T^{-1}$). $\Delta G'$ of these reactions becomes zero when the $PH_2$ is 76 Pa and the formate concentration is 32 $\mu$M, assuming that $[NAD^+]=[NADH]$ and $[HCO_3^-]=25$ mM. Fig.2 shows the effects of $H_2$ (A) and formate (B) on the Gibb's free energy change of electron transfer components when coupled to $H_2$ and formate production, respectively.

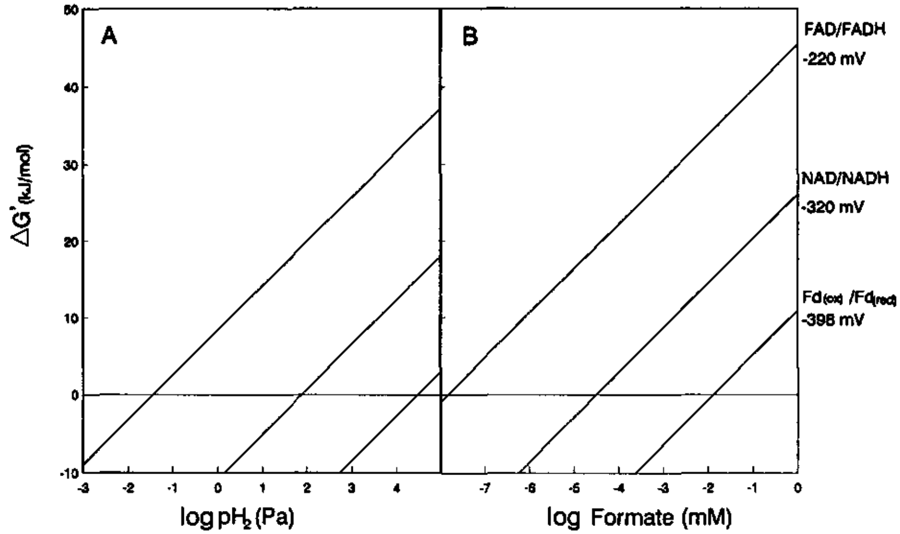

Fig. 2. Diagram of the dependence of the Gibb's free energy changes of the redox couples prevailing in biological systems on the hydrogen partial pressure (A) and the formateconcentration (B) when the oxidations are coupled to proton reduction (A) and to bicarbonate (B) reduction.

table 2. Redox potentials of the redox couples possibly involved in the anaerobic degradation of propionate and butyrate, and their free energy changes when coupled to proton reduction ($\Delta G^{\circ\prime}_1$) or when coupled to bicarbonate reduction ($\Delta G^{\circ\prime}_2$)[a]

| Redox couples | E°'(mV) | $\Delta G^{\circ\prime}_1$(kJ/mol) | $\Delta G^{\circ\prime}_2$(kJ/mol) |
|---|---|---|---|
| Acetyl-CoA/pyruvate | -490 | -12.2 | -13.5 |
| $CO_2$/formate | -432 | +1.3 | |
| $H^+/H_2$ | -414 | | -1.3 |
| Fd(ox)/Fd(red) | -398 | +3.1 | +1.7 |
| $NAD(P)^+/NAD(P)H$ | -320 | +18.1 | +16.8 |
| $FAD/FADH_2$ | -220 | +37.4 | +36.1 |
| Acetoacetyl-CoA/3-OH-butyryl-CoA | -190 | +31.6 | +30.3 |
| Oxaloacetate$^{2-}$/malate$^{2-}$ | -172 | +47.7 | +46.4 |
| Crotonyl-CoA/butyryl-CoA | -126[b] | +75.2 | +73.9 |
| Fumarate$^{2-}$/succinate$^{2-}$ | +33 | +86.2 | +84.7 |

a: Except the data from references b, all the data of E°'are taken from Thauer et al. (1977). The $\Delta G^{\circ\prime}$ values are calculated according to the equation $\Delta G^{\circ\prime} = -n.F.\Delta E^{\circ\prime}$.

b: Gustafson et al. (1986).

## Hydrogen flux and formate flux in syntrophic cultures

As syntrophic reactions are completed by more than one organism, interspecies mass transfer is essential. The conversion rates are determined by the flux (J) of $H_2$ or formate in cultures. The flux depends on the diffusion coefficient (D), the $H_2$ and formate concentration gradients (C), the diffusion distance (d) between $H_2$/formate-producing and $H_2$/formate-consuming cells, and the surface area of the $H_2$/formate-producing organism (A). Fluxes can be described by a simple Fick's diffusion equation (Schink & Thauer 1988).

$$J = -A \cdot D \cdot \frac{C_2 - C_1}{d} mol \cdot \sec^{-1}$$

Aggregation and juxtaposition of bacterial communities appears to be very important in syntrophic degradation. If the different bacteria are in the close vicinity of each other the diffusion distance is minimized. The high rates of $CH_4$ formation in UASB reactors are achieved because they contain granular sludge with a high biomass density. Disruption of the structure of methanogenic granules reduced the propionate degradation rate by 90 % (Grotenhuis et al. 1991). Other examples are also described, e.g. the degradation rates of butyrate and propionate decreased 35 and 25% after disintegration of the thermophilic granules (Schmidt & Ahring 1993), separating ethanol-oxidizing and $H_2$-utilizing bacteria by a dialysis membrane led to an increase in the doubling time by 7 to 11 hours (Stieb & Schink 1987), while shortening the interbacterial distances in syntrophic cultures increased the $CH_4$ formation rate from propionate (Stams et al. 1992) and from butyrate (Dwyer et al. 1988; Schmidt & Ahring 1993).

The steepness of the $H_2$ and formate concentration gradient is another parameter which affects the fluxes. As described above the maximal $H_2$ and formate concentration that can be reached by acetogens depends on the thermodynamics of the conversions (Stams 1994). The same is true for the threshold values for formate and $H_2$ that can be reached by $H_2$/formate-utilizing anaerobes. The threshold values for $H_2$ of methanogens and sulfate reducers are 3-16 and 1-3 Pa, respectively (Cord-Ruwisch et al. 1988; Dwyer et al. 1988; Boone et al. 1989; Stams et al. 1992; Boone & Bryant 1980). The maximum specific growth rates of propionate-, butyrate- and benzoate-degrading acetogenic bacteria in coculture with methanogens were 0.10, 0.19 and 0.10 $day^{-1}$, respectively; while in cocultures with sulfate reducers the growth rates were 0.19, 0.31 and 0.13 $day^{-1}$, respectively (Boone & Bryant 1980; McInerney et al. 1979; Mountfort & Bryant 1982). These differences were explained by the differences in $H_2$ gradients (Dolfing 1988).

## Interspecies $H_2$ transfer

Since it was discovered that *Methanobacillus omelianskii* consisted of an ethanol-oxidizing bacterium (S organism) and *Methanobacterium bryantii*, Bryant et al. (1967) assumed that $H_2$ transfer could play a role in syntrophic degradation. Hungate (1967) was the first to demonstrate that $H_2$ was an intermediate in fermentative processes in the rumen. This has led to the development of a 3-step model for the anaerobic mineralization of organic matter, and the concept of interspecies $H_2$ transfer was developed by Wolin (1976; 1982). Convincing evidences for the role of $H_2$ were provided for those syntrophic consortia in which only $H_2$-scavengers are present, such as in the classical ethanol-oxidizing *M. omelianskii* culture (Bryant et al. 1967), a butyrate-degrading culture with *S. wolfei* (McInerney et al. 1979; 1981), a thermophilic butyrate-oxidizing culture (Henson & Smith 1985) and a thermophilic propionate-oxidizing culture (Stams et al. 1992). Indeed in such syntrophic cocultures $H_2$ partial pressures could be measured close to those which can be calculated from thermodynamic considerations. In a syntrophic ethanol-oxidizing culture an equilibrium $H_2$ partial pressure in the range of 500-2000 Pa was measured (Stieb & Schink 1987; Seitz et al. 1988). Whereas the $H_2$ partial pressure was only at 30-34 Pa in a thermophilic propionate-oxidizing coculture (Stams et al. 1992) and 95 Pa in a butyrate-oxidizing culture (Dwyer et al. 1988). Moreover, inhibition of propionate and butyrate oxidation by addition of $H_2$ was observed in mesophilic propionate-acclimatized sludge and in a thermophilic butyrate-oxidizing culture, respectively (Fukuzaki et al. 1990; Ahring & Westermann 1988).

## Interspecies formate transfer

Formate as an alternative electron carrier during syntrophic degradation was proposed for the first time by Bryant et al. (1967), and later on by McInerney et al. (1979) and Boone & Bryant (1980). However, Thiele & Zeikus (1988) proposed that formate transfer might even be more important than $H_2$ transfer. It was observed that the minimum threshold levels for $H_2$ of the methanogens prevailing in some anaerobic ecosystems were about 6 times higher than the $H_2$ gas levels observed in such environments (Thiele et al. 1988), and that the turnover of the $H_2$ pool could only account for 5-10% to the measured electron transfer rates. Thiele & Zeikus (1988) put forward the interspecies formate transfer theory based on that: i) many $H_2$-consumers in syntrophic cultures can use formate; ii) the redox potential of the couple $H^+/H_2$ and $HCO_3^-$/formate are nearly same; iii) some acetogenic bacteria are able to form formate during syntrophic degradation (Hungate 1967; Thiele & Zeikus 1988).

Although *S. wolfei* degraded butyrate in coculture with *Methanobacterium bryantii*, the growth rate and $CH_4$ production were increased significantly in the coculture with *M. hungatei* (McInerney et al. 1979). *M. hungatei* is often abundantly present in many syntrophic consortia (table 1), and formate transfer clearly occurred in a coculture with *Desulfovibrio baarsii* (Zindel et al. 1988), a bacterium which utilizes only formate but not $H_2$. Direct experimental evidence for formate formation was obtained for the syntrophic conversion of ethanol to $CH_4$ in microbial flocs of a reactor treating whey (Thiele & Zeikus 1988). Formate accumulated when methanogenesis in the flocs was inhibited and formate formation was dependent on $CO_2$ and ethanol but not on $H_2$. Other evidence for formate transfer was obtained with a coculture of *S. wolfei* and *M. formicicum* by Boone et al. (1989) using a diffusion model. They calculated that the formate diffusion rate could account for the $CH_4$ formation rate in the culture but the diffusion rate of $H_2$ could not. Thiele et al. (1988) calculated that the total ratio of the dissolved formate and $H_2$ concentration should be between 10 and 200 in pure and mixed cultures of fermentative or acetogenic bacteria and that under equilibrium conditions in anaerobic ecosystems and at $HCO_3^-$ levels above 15 mM, more than 95% of dissolved reducing equivalents would occur as formate due to its higher solubility than $H_2$. However, Schink (1992) argued that $H_2$ and formate besides through water have to be transported through the cell membranes of the acetogen and the methanogen. Therefore, interspecies $H_2$ transfer might be more favorable because $H_2$ but not formate has a good solubility in the lipophilic phase.

So far, it is not clear yet whether $H_2$ or formate transfer is more important during syntrophic degradation, this especially because some of the above mentioned observations can be explained in other ways. E.g. $H_2$ or formate formation in "inhibited" cultures is not a clear proof for $H_2$ or formate transfer because methanogens and some acetogens are able to interconvert $H_2/CO_2$ and formate (Wu et al. 1993; this thesis). It might also be that $H_2$ transfer and formate transfer occur concomitantly in one system (Schink 1992); or that $H_2$ transfer is more important in aggregated structures while formate transfer is important in suspended cultures (Stams 1994). For, *Methanobrevibacter*, a methanogen which uses only $H_2/CO_2$, is the most numerous methanogen in propionate-degrading granular sludge (Grotenhuis et al. 1991; Stams et al. 1989); while this methanogen is unable to drive the degradation of butyrate and propionate in suspended culture by *S. bryantii* and MPOB, respectively (this thesis).

### Interspecies acetate transfer

Acetate is one of the products in syntrophic degradation, and also an important precursor of methanogenesis. It contributes for 70% of the $CH_4$ formation in anaerobic digestors degrading organic matter (Mah et al. 1990; Gujer & Zehnder 1983). Acetate removal by methanogens can affect the energetics of various reactions to different extents. Low acetate concentrations are absolutely required in isovalerate oxidation, where 3 moles of acetate and only 1 mol of $H_2$ are formed. Theoretically, $H_2$ removal by methanogens can change the Gibb's free energy change of the overall reaction in isovalerate oxidation from +20.2 kJ/mol isovalerate (Eq. 3) to -13.6 kJ/mol (Eq. 4); while acetate removal by methanogens can result in a Gibb's free energy change of -71.6 kJ/mol (Eq. 5). The addition of 1 mM acetate prevented this methanogenic coculture from growth and degrading isovalerate in a fresh subculture (Stieb & Schink 1986).

$\Delta G^{o\prime}$(kJ/mol)

$$C_5H_9O_2^- + HCO_3^- + H_2O \rightarrow 3C_2H_3O_2^- + H_2 + H^+ \qquad +20.2 \ (3)$$

$$C_5H_9O_2^- + 5/4HCO_3^- + 1/2H_2O \rightarrow 3C_2H_3O_2^- + 1/4CH_4 + 3/4H^+ \qquad -13.6 \ (4)$$

$$C_5H_9O_2^- + 4H_2O \rightarrow 3CH_4 + H_2 + H^+ + 2HCO_3^- \qquad -71.6 \ (5)$$

Acetate is the only intermediate transferred in the methanogenic degradation of acetone (Platen & Schink 1987), and *Methanothrix* sp. is the only methanogen in the coculture. Inhibition of methanogenesis by bromoethane sulfonic acid (BrES) or ethylene led to acetate accumulation, while the acetone degradation rate decreased. Although the oxidation of acetone to acetate is exergonic (Eq. 6), acetate removal obviously was essential for energy conservation during acetone conversion (Eq. 7).

$\Delta G^{o\prime}$(kJ/mol)

$$CH_3COCH_3 + HCO_3^- \rightarrow 2CH_3COO^- + H^+ \qquad -34.2 \ (6)$$

$$CH_3COCH_3 + 2H_2O \rightarrow 2CH_4 + H^+ + HCO_3^- \qquad -89.2 \ (7)$$

Acetate accumulation also retards propionate and butyrate degradation in syntrophic consortia. Addition of 3.3 mM acetate decreased the propionate consumption by 60% in methanogenic sludge (Gorris et al. 1989) while addition of *Methanosarcina* to the coculture of *S. wolfei* and *M. hungatei* increased the butyrate degradation rate by 30% (Beaty & McInerney 1989). These observations suggest that acetate transfer can occur either together with $H_2$/formate transfer or independently.

## Propionate oxidation

Propionate is an important intermediate in methanogenic mineralization of organic matter. It accounts for 35 % to the methane formation in a digestor fermenting animal waste (Mah et al. 1990). Complete mineralization of propionate into methane and $CO_2$ will liberate 56.6 kJ. This is about the amount needed to synthesize 1 mol of ATP (Schink 1992).

$$Propionate^- + 1.75H_2O \rightarrow 1.75CH_4 + 1.25HCO_3^- + 0.25H^+ \qquad \Delta G^{o\prime} = -56.6 kJ/mol$$

This process is performed by 3 types of bacteria; acetogenic bacteria degrade propionate into $H_2$/formate and acetate, which then are converted into methane by $H_2$/formate-utilizing and aceticlastic methanogens, respectively. Therefore, the energy which is conserved during propionate oxidation has to be shared by the 3 kinds of bacteria.

So far, only a few of propionate-oxidizing cultures are documented. *Syntrophobacter wolinii* is the first propionate oxidizer which was enriched from a methanogenic enrichment from an anaerobic municipal sewage digestor and was obtained in a defined coculture with a *Desulfovibrio* sp. (Boone & Bryant 1980). Only very recently a binary culture of *S. wolinii* and *M. hungatei* was obtained (Dörner 1992). Stams et al. (1993) described another mesophilic propionate-oxidizing bacterium (MPOB) in coculture with a *Methanospirillum* sp.. They succeeded to get a methanogen-free culture by growing it on fumarate. In the absence of methanogens this bacterium is able to i) reduce fumarate to succinate with $H_2$ or formate as electron donors, ii) oxidize propionate with fumarate as electron acceptors, iii) ferment fumarate to succinate and $CO_2$. 16S rRNA sequence analysis revealed that *S. wolinii* and MPOB were phylogenetically related with sulfate-reducing bacteria (Harmsen et al. 1993; 1994), and indeed these two bacteria are able to couple propionate oxidation to sulfate reduction (Dörner 1992; Van Kuijk unpublished results). Some other more or less defined syntrophic propionate-oxidizing cultures have been obtained (Koch et al. 1983; Mucha et al. 1988; Robbins 1988; Wu et al. 1992; Dörner 1992; Stams et al. 1992).

Experiments with $^{14}C$ and $^{13}C$ labelled propionate revealed that a randomizing pathway is used for propionate oxidation (Koch et al. 1983; Houwen et al. 1987, 1991; Robbins 1988). These findings led to propose the propionate oxidation pathway as given in Fig. 3,

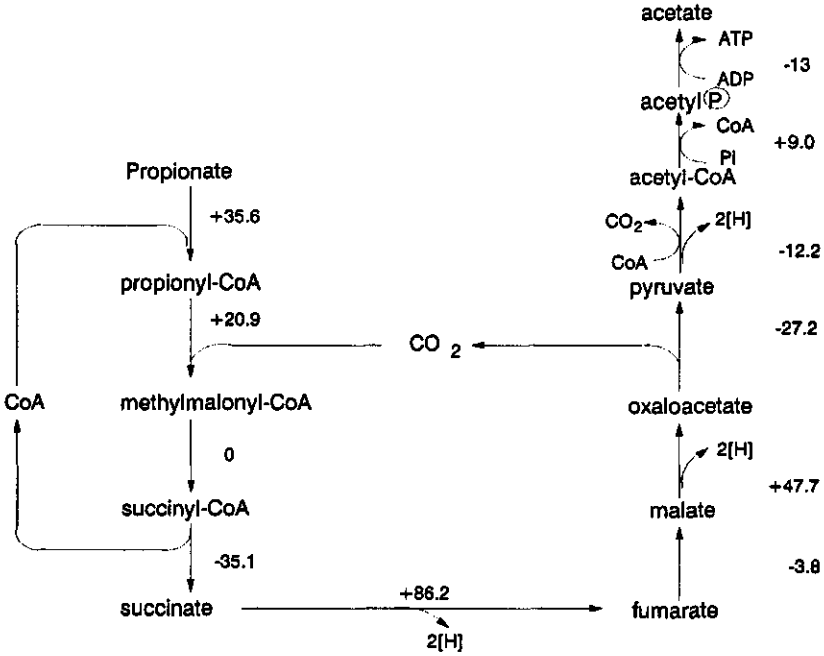

Fig. 3. Pathway of syntrophic propionate oxidation and the $\Delta G^{\circ\prime}$ values (kJ/mol) of the intermediate steps when the oxidation reactions are coupled to proton reduction.

which was similar to that found in *Desulfobulbus propionicus* (Stams et al. 1984). Enzyme measurement in cell extracts of *S. wolinii* (Houwen et al. 1990) and MPOB (Plugge et al. 1993) confirmed that methylmalonyl-CoA pathway is involved in syntrophic propionate oxidation. However, the difference between the two organisms is that propionate is activated to propionyl-CoA via a propionate kinase in *S. wolinii*, while the activation is achieved via a propionate:acetyl-CoA HS-CoA transferase in MPOB. The oxidation of 1 mol of propionate would yield 1 ATP by substrate level phosphorylation. During propionate oxidation, reducing equivalents are released in the oxidations of succinate to fumarate, malate to oxaloacetate and pyruvated to acetyl-CoA. The midpoint redox potential of the redox couple succinate/fumarate is +30 mV. While the redox potentials of $H^+/H_2$ and $HCO_3^-/HCOO^-$ are -414 mV and -432 mV, respectively. To make succinate oxidation coupled to $H_2$ or formate formation feasible, $H_2$ or formate levels of $10^{-9}$ Pa and $10^{-8}$ $\mu$M would be required. However, these values are far lower than can be created by $H_2$/formate-utilizing methanogens. Therefore, a reversed electron transport mechanism has to be proposed. It has been speculated that 2/3 ATP would be invested to drive this reversed electron transport (Stams 1994). Evidence for the existence of a reversed electron transport mechanism was obtained with a syntrophic glycolate-oxidizing bacterium (Friedrich et al. 1991; Friedrich & Schink 1993), a butyrate oxidizer-*Syntrophomonas wolfei* (Wallrabenstein & Schink 1994) and an acetate-oxidizing sulfur-reducing *Desulfuromonas acetoxidans* (Paulsen et al. 1986).

## Butyrate oxidation

Butyrate is another important intermediate during mineralization of biopolymers in methanogenic environments. It accounts for 8% of the total methane formation in a digestor fermenting animal waste (Mah et al. 1990). As with propionate conversion, mineralization of butyrate into methane and $CO_2$ also requires 3 different types of bacteria, viz butyrate-oxidizing acetogens, $H_2$/formate-utilizing and aceticlastic methanogens. The overall equation for butyrate conversion is :

$$Butyrate^- + 2.5H_2O \rightarrow 2.5CH_4 + 1.5HCO_3^- + 0.5H^+ \qquad \Delta G^{\circ\prime} = -81.7 kJ/mol$$

Schink & Thauer (1988) assumed that the free energy for each partial reaction of butyrate oxidation is about -20 kJ, when taking the concentrations of relevant reactants at the conditions prevailing in methanogenic digestors into account. As with propionate oxidation 20 kJ of free energy is available for each of the bacteria.

Several butyrate oxidizers have been isolated, and they are the best characterized syntrophs so far. Based on 16S rRNA sequences, a new family, *Syntrophomonadaceae* (Zhao et al. 1993), was created. *Syntrophomonas wolfei* (McInerney et al. 1979; 1981) is the first characterized butyrate-oxidizing bacterium. It is a nonsporulating gram-negative mesophilic anaerobe, which degrades $C_4$-$C_{18}$ straight-chain fatty acids in syntrophic association with methanogens. *Syntrophospora bryantii* (former *Clostridium bryantii*) (Stieb & Schink 1985; Zhao et al. 1990), is a spore-forming butyrate degrader, which could be isolated from marine and fresh water mud samples. It oxidizes fatty acids of up to 10 carbon atoms. Some other butyrate-oxidizers were also described such as strains SF-1, NSF-1 (Shelton & Tiedje 1984), BH (Wu et al. 1992), *Syntrophomonas sapovorans* (Roy et al. 1986) and thermophilic bacteria in syntrophic association with *Methanobacterium thermoautotrophicum* (Ahring & Westermann 1987; Henson & Smith 1985). An isobutyrate-oxidizing strain was described in a triculture with *Methanobacterium formicicum* and *Methanosarcina mazei* (Jain et al. 1989). It reversibly isomerizes isobutyrate to butyrate and degrades isobutyrate to acetate via butyrate.

As far as it has been investigated, these syntrophs can grow in pure culture on unsaturated short-chain fatty acids. The pure cultures of *S. wolfei* (Beaty & McInerney

1987) and *S. bryantii* (Zhao et al. 1990) were obtained by growth on crotonate. Both bacteria ferment crotonate to butyrate and acetate. *S. wolfei* is able to couple butyrate oxidation to the reduction of unsaturated alkanes (Kasper et al. 1987), while *S. bryantii* is able to couple butyrate oxidation to the reduction of pentenoate to valerate (Chapter 3, this thesis).

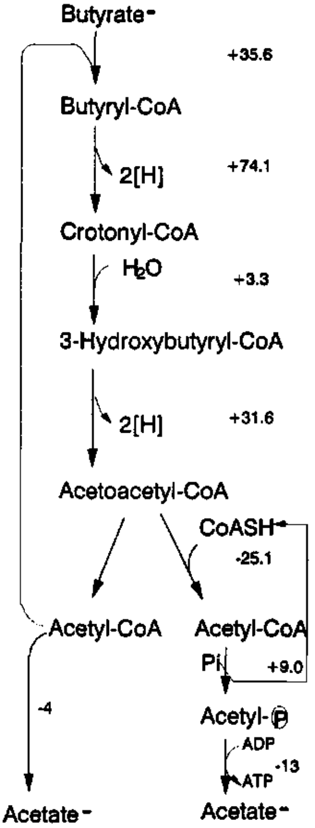

Fig. 4. Pathway of syntrophic butyrate oxidation and the $\Delta G^{\circ\prime}$ values (kJ/mol) of the intermediate steps when the oxidations are coupled to proton.

Syntrophic butyrate oxidation proceeds through the crotonyl-CoA pathway (Fig. 4). This has been demonstrated for *S. wolfei* (Wofford et al. 1986) and for *S. bryantii* (Schink 1992). Butyrate is activated to butyryl-CoA via a butyrate:acetyl-CoA HSCoA transferase. Two acetate are formed as end products and reducing equivalents are released in the oxidation of butyryl-CoA to crotonyl-CoA and of 3-hydroxy-butyryl-CoA to acetoacetyl-CoA. These reducing equivalents are formed at the level of $FADH_2$ and NADH, respectively. One ATP is synthesized by substrate level phosphorylation in the acetate kinase reaction. Because the reducing equivalents are formed at redox potential of -126 mV (butyryl-CoA dehydrogenase) and -190 mV (3-OH-butyryl dehydrogenase), for butyryl-CoA oxidation, $H_2$ and formate levels as low as $10^{-8}$ Pa and $10^{-7}$ $\mu$M are required, when the electrons are disposed as $H_2$ and formate, respectively. Thauer & Morris (1984) speculated that butyrate-oxidizers spend part of the ATP formed by substrate level phosphorylation to drive a reversed electron transport reaction to release the electron at a lower redox potential. The butyryl-CoA dehydrogenase and hydrogenase of *S. wolfei* were demonstrated to be partly membrane-bound (Dörner 1992; Wallrabenstein & Schink 1994). Moreover, $H_2$ formation could be inhibited by a protonophore (CCCP) or by an ATPase inhibitor (DCCD). These observations support the existence of a reversed electron transfer mechanism in butyrate oxidizing bacteria.

## Outline of the thesis

For clarifying the relative importance of $H_2$ and formate transfer during syntrophic degradation of propionate and butyrate, a propionate-oxidizer (MPOB) and a butyrate-oxidizer *S. bryantii* were used.

Pure cultures of the acetogens offer the ability to construct artificial defined methanogenic cocultures. Chapter 2 deals with propionate degradation in cocultures of MPOB with different methanogens, and the relative importance of $H_2$ and formate is deduced by determining the degradation kinetics in the different cocultures. Moreover, the requirement of low formate concentration during syntrophic propionate oxidation is also determined in a triculture of MPOB, a formate-cleaving bacterium and a $H_2$-consuming methanogen. Chapter 3 describes butyrate degradation by *S. bryantii* in different methanogenic cocultures and tricultures, and in single culture with pentenoate as electron acceptor. The effect of acetate removal on degradation of propionate and butyrate was determined as well (Chapter 2 and 3). Chapter 4 provides evidence for the formation of both $H_2$ and formate during butyrate oxidation by *S. bryantii* and during propionate oxidation by MPOB. The effect of $HCO_3^-$ on $H_2$ and formate formation is also described in this chapter.

The localization of the enzymes responsible for $H_2$ and formate production in the acetogens and the kinetic properties of hydrogenase and formate dehydrogenase are determined in *S. bryantii* and MPOB. These results are presented in Chapter 5 and 6, respectively. Finally, in chapter 7 the observations presented in this thesis are summarized.

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## Chapter 2

# Anaerobic degradation of propionate by a mesophilic acetogenic bacterium in coculture and triculture with different methanogens

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## ABSTRACT

A mesophilic acetogenic bacterium (MPOB) oxidized propionate to acetate and $CO_2$ in cocultures with the formate- and hydrogen-utilizing methanogens *Methanospirillum hungatei* and *Methanobacterium formicicum*. Propionate oxidation did not occur in cocultures with two *Methanobrevibacter* strains, which grew only with hydrogen. Tricultures consisting of MPOB, one of the *Methanobrevibacter* strains and organisms which are able to convert formate into $H_2/CO_2$ (*Desulfovibrio* G11 or a homoacetogenic bacterium EE121) also degraded propionate. MPOB, in the absence of methanogens, was able to couple propionate conversion to fumarate reduction. This propionate conversion was inhibited by hydrogen and by formate. Formate and hydrogen blocked the energetically unfavorable succinate oxidation to fumarate involved in propionate catabolism. Low formate and low hydrogen concentrations are required for the syntrophic degradation of propionate by MPOB. In triculture with *M. hungatei* and the aceticlastic *Methanothrix soehngenii*, propionate was degraded faster than in biculture with *M. hungatei*, indicating that low acetate concentrations are favorable for propionate oxidation as well.

## INTRODUCTION

Propionate is an important intermediate in the degradation of organic matter under methanogenic conditions. It may account for 35% of the total methanogenesis in digestors (17). Propionate oxidation is accomplished by syntrophic consortia of acetogenic and methanogenic bacteria (2, 6, 17, 25). Due to unfavorable energetics propionate oxidation to acetate is only possible when the products hydrogen and/or formate (Table 1; equation 1 and 2) are kept low by methanogens (Table 1; equation 3 and 4). At present, the relative importance of formate and hydrogen in syntrophic degradation is not clear. Several syntrophic cocultures have been described in which only $H_2$-consuming methanogens were present (2, 16, 25, 26). In these cocultures $H_2$ inhibited the degradation (1, 22). However, some observations made in mixed populations could not easily be explained by interspecies hydrogen transfer. Thiele and Zeikus (30) put forward that interspecies formate transfer might be important as well. Boone et al (3) investigated a butyrate-oxidizing coculture of *Syntrophomonas wolfei* and *Methanobacterium formicicum* and calculated by using a formate and hydrogen diffusion model that the high methane formation rate could not be explained by interspecies $H_2$ transfer, but it could be explained by interspecies formate transfer. As $H_2$ and formate concentrations in methanogenic ecosystems are extremely low, direct evidence for the quantitative importance of $H_2$ or formate transfer is difficult to obtain in mixed populations.

Recently, we succeeded in growing a mesophilic propionate-oxidizing bacterium (MPOB)no fumarate in the absence of methanogens (27). The bacterium is able to oxidize propionate to acetate coupled to the reduction of fumarate to succinate. In addition, the bacterium can ferment fumarate to succinate and $CO_2$ or reduce fumarate to succinate with hydrogen and formate as electron donors. The ability to grow in the absence of methanogens offered the ability to construct different defined co- and tricultures. By using methanogens that can use both hydrogen and formate and methanogens that can only use hydrogen, the role of hydrogen and formate in the syntrophic degradation of propionate could be studied directly. In this investigation we also used organisms that are able to interconvert hydrogen and formate (Table 1, equation 6). For this purpose we chose a homoacetogen and *Desulfovibrio* sp. The effect of the presence of the aceticlastic *Methanothrix soehngenii* on syntrophic degradation of propionate was studied as well.

Table 1. Reactions possibly involved in the syntrophic degradation of propionate

| Reaction equations | $\Delta G^{\circ\prime}$(kJ/mol) |
|---|---|
| Propionate oxidation: | |
| 1. $CH_3CH_2COO^- + 3H_2O$ ---> $CH_3COO^- + HCO_3^- + 3H_2 + H^+$ | +76.5 |
| 2. $CH_3CH_2COO^- + 2HCO_3^-$ ---> $CH_3COO^- + 3HCOO^- + H^+$ | +72.4 |
| Methanogenesis: | |
| 3. $4H_2 + HCO_3^- + H^+$ ---> $CH_4 + 3H_2O$ | -135.6 |
| 4. $4HCOO^- + H^+ + H_2O$ ---> $CH_4 + 3HCO_3^-$ | -130.1 |
| 5. $CH_3COO^- + H_2O$ ---> $CH_4 + HCO_3^-$ | -31 |
| Formate conversion: | |
| 6. $HCOO^- + H_2O$ ---> $H_2 + HCO^-_3$ | +1.3 |

$\Delta G^{\circ\prime}$ values were taken from Thauer et al 1977 (29).

## MATERIAL AND METHODS

**Organisms.** The mesophilic propionate-oxidizing bacterium (MPOB) was described before (21, 27). *Methanospirillum hungatei* DSM 864 (8), *Methanobacterium formicicum* DSM 1535 (5), and the *Methanobrevibacter arboriphilus* strains DSM 1125 (32) and DSM 744 (31) were purchased from the German Collection of Microorganisms (Braunschweig, Germany). *Methanothrix soehngenii* (*Methanosaeta concilii*) (13) was from our culture collection. *Desulfovibrio* G11 (2, 18) was isolated by us from a coculture with *Syntrophobacter wolinii* DSM 2805. The homoacetogenic bacterium EE121 was isolated at our laboratory (20).

**Media and cultivation.** MPOB was routinely grown in a mineral medium containing sodium fumarate (6.5 g/l) as sole carbon and energy source under a gas phase of 162 kPa $N_2/CO_2$ (80/20) (27). Except for *Methanothrix soehngenii*, all methanogens, *Desulfovibrio* G11 and the homoacetogen EE121 were cultivated in a basal mineral medium with a composition as described previously (10). However, the trace minerals $Na_2SeO_3$ and $Na_2WO_4$ were omitted, and 0.25 g cysteine, 0.25 g yeast extract, 0.25 g biotrypticase and 0.16 g sodium acetate were added per liter medium. The gas phase was 162 kPa $H_2/CO_2$ (80/20). For the routine growth of *Desulfovibrio* G11, 2.84 g/liter sodium sulfate was added to the medium. The homoacetogen EE121 was routinely grown on glucose (5 g/l) under a gas phase of $N_2/CO_2$ (80/20). For the triculture experiment, *Desulfovibrio* G11 and homoacetogen EE121 were grown in the media containing 1.7 g/l formate under $N_2/CO_2$ (80/20). *Methanothrix soehngenii* was grown in the same medium as MPOB except that a tenfold higher concentration of vitamins and a twofold higher concentration of trace elements were used; sodium acetate (5 g/l) was the carbon source. All the bacteria were cultivated in 50 ml medium in 120-ml serum vials (Aluglas Verenigde Bedrijven B. V., Amersfoort, the Netherlands) which were closed with butyl rubber stoppers (Rubber B.V., Hilversum, the Netherlands) and aluminum caps. The incubation temperature was 36±1°C.

**Propionate oxidation by bi- and tri-cultures.** Bacterial cultures were grown in the media described above and when growth had ceased, cocultures were constructed by mixing aseptically the MPOB culture and the different hydrogen- and acetate-degrading cultures. The inoculation sizes are given below. Unless stated otherwise, propionate was added as carbon and energy source, and the gas phase was $N_2/CO_2$ (80/20). At different time intervals gas and liquid samples were taken and analyzed. Propionate degrading rates were estimated from the linear part of the propionate degradation curves.

**Effect of $H_2$ and formate on the growth of MPOB in the absence of methanogens.** MPOB was cultivated in six 120-ml serum vials containing 50 ml mineral medium with 40 mM fumarate and 20 mM propionate as substrates and a gas phase of $N_2/CO_2$ (80/20). After about one month of incubation, two of the vials were flushed with 162 kPa $H_2/CO_2$ (80/20), whereas to two other vials formate (20 mM, final concentration) was added. Two vials served as controls. Incubation was continued, and after various periods of time samples were taken and analyzed.

**Analytical methods.** Methane and hydrogen were measured on a Packard-Becker 417 gas chromatograph as described before (11). Formate, malate, fumarate, succinate, acetate and propionate were measured by HPLC (7).

## RESULTS

**Propionate degradation by cocultures and tricultures with different methanogens.** To t the ability of different methanogens to act as the syntrophic partner organism in the syntrophic degradation of propionate, 10 ml of a fumarate-grown culture of MPOB was added to 40 ml of a hydrogen-grown culture of the methanogens. The gas phase was changed to $N_2/CO_2$ and propionate was added (15 mM). The cocultures of MPOB with *M. hungatei* (Fig. 1A) and *M. formicicum* (Fig. 1B) degraded propionate to acetate and methane. The propionate degradation rates were about 0.8 mM/day and 0.4 mM/day, respectively. The methane yield was somewhat lower than expected because some fumarate (2-3 mM) was still present in the cocultures. The cocultures of MPOB with *M. hungatei* and *M. formicicum* could be subcultured by transfer in fresh media with propionate as the sole substrate. Propionate degradation was not observed in the cocultures of MPOB with *M. arboriphilus* DSM 1125, *M. arboriphilus* DSM 744 and *M. soehngenii* (results not shown). These incubations were continued for more than two months, but even after that period of time no propionate degradation had occurred. To test whether the distance between MPOB and the $H_2$-consuming methanogen is of importance, bacteria were immobilized. By the addition of $FeCl_2$ (2 mM, final concentration), the sulfide which was present in the media precipitated

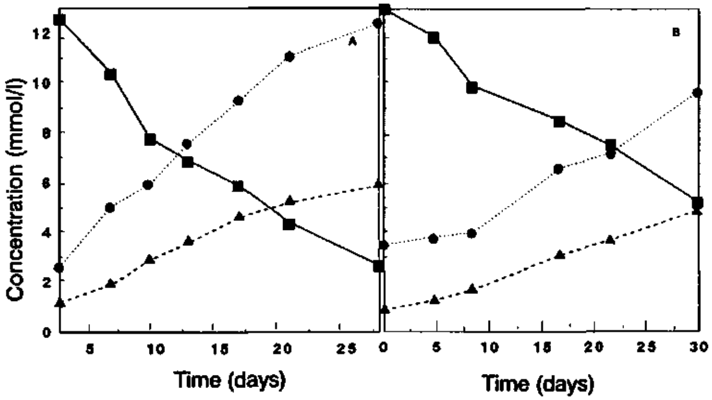

FIG. 1. Propionate (■) degradation, and acetate (●) and methane (▲) production by MPOB in coculture with *Methanospirillum hungatei* DSM 864 (A) and *Methanobacterium formicicum* DSM 1535 (B).

as FeS. During precipitation anaerobic bacteria co-precipitated leading to high cell densities within the precipitates (26). Also under these conditions propionate was only degraded in cocultures with *M. hungatei* and *M. formicicum*. The degradation rates were almost the same as observed in the suspended cocultures (data not shown).

The effect of the additional presence of an acetate-degrading methanogen is shown in Figure 2. The methanogenic triculture was constructed by mixing 20 ml *M. soehngenii* culture, 20 ml *M. hungatei* culture, and 10 ml MPOB culture. The biculture was constructed as described above. In the triculture 21 mM propionate was degraded and 28.3 mmol/l methane was produced within 18 days. The degradation rate was about 1.4 mM/day. Tricultures in which *M. hungatei* was replaced by one of the *Methanobrevibacter* strains did not degrade propionate (results not shown).

**Propionate degradation by tricultures consisting of MPOB, a $H_2$-utilizing methanogen and a formate cleaving bacterium.** *Desulfovibrio* in the absence of sulfate (9) is able to

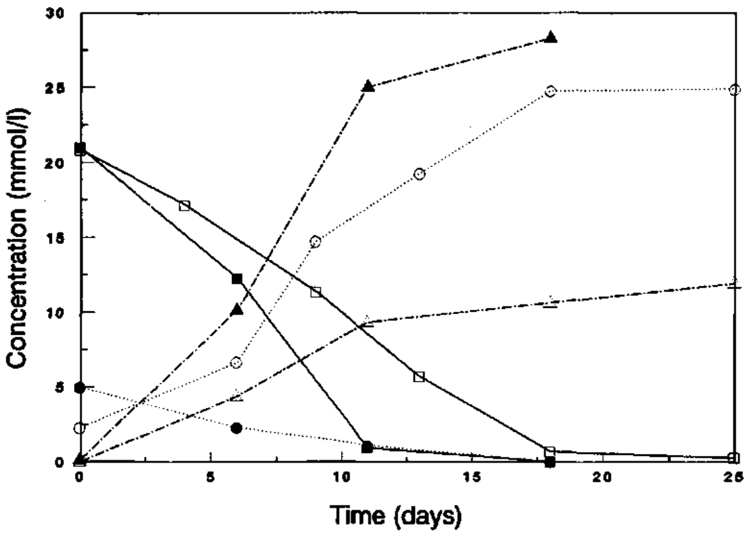

FIG. 2. Propionate (■,□) degradation, and acetate (●,○) and methane (▲,△) production by MPOB in triculture with *Methanospirillum hungatei* and *Methanothrix soehngenii* (closed symbols) and in biculture with *Methanospirillum hungatei* (open symbols).

cleave formate into $H_2$ and $CO_2$ (Table 1, equation 6) and a homoacetogenic bacterium EE121 (20) is able to convert formate partly into $H_2/CO_2$ and partly into acetate. Because the $\Delta G^{\circ\prime}$ of formate cleavage is around zero, a complete conversion of formate to $H_2$ is only possible when the $H_2$ is taken away by a $H_2$-utilizing methanogen. A possible role of formate in propionate oxidation can be demonstrated when propionate oxidation can be coupled to a $H_2$-consuming methanogen in the presence of a bacterium able to cleave formate at a high rate. *Desulfovibrio* G11 in the absence of sulfate cleaved about 4 mM formate into 4 mM $H_2$, and the homoacetogen EE121 converted 4 mM formate into about 2 mmol/l $H_2$ in 7 days, the remainder presumably being converted to acetate. After the addition of *M. arboriphilus* (DSM 1125) a much faster degradation of formate was observed, about 17 mM in 7 days. A triculture was constructed by mixing 30 ml of a formate-pregrown culture of *Desulfovibrio*

G11 or the homoacetogen EE121, 10 ml *M. arboriphilus* DSM1125 and 10 ml MPOB. Figure 3 shows that propionate was degraded in the tricultures, whereas in the cocultures with either the homoacetogen EE121, *Desulfovibrio* G11 or *M. arboriphilus* alone no degradation of propionate was observed (results not shown). In the triculture with *Desulfovibrio* G11 the propionate concentration had decreased by about 6 mM in 2 months

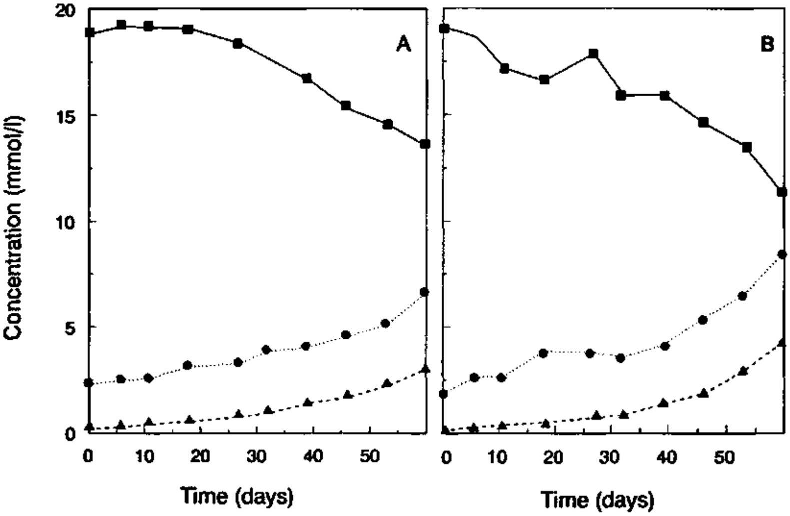

FIG. 3. Propionate (■) degradation and acetate (●) and methane (▲) production by tricultures of MPOB, *Methanobrevibacter arboriphilus* DSM 1125, and *Desulfovibrio* strain G11 (A) and MPOB,*Methanobrevibacter arboriphilus* DSM 1125, and the homoacetogenic bacterium EE121 (B).

(Fig.3A), which corresponded with a degradation rate of about 0.15 mM/day. In the triculture with the homoacetogen EE121 a somewhat faster propionate degradation was observed; the propionate concentration decreased by 8 mM in 2 months (Fig. 3B), corresponding to a rate of 0.23 mM/day.

Effect of $H_2$ and formate on propionate degradation by MPOB in the absence of methanogen.

To study the effect of hydrogen and formate on propionate degradation in the absence of methanogens, 162 kPa $H_2/CO_2$ or 18 mM formate were added to MPOB growing on fumarate + propionate. Fig. 4A shows that propionate degradation was totally inhibited by both $H_2$ and formate, and acetate was even converted back to propionate. Fumarate conversion was not affected either in the culture with $H_2$ or in the culture with formate (Fig. 4B). As described previously, the ratio of fumarate degraded to succinate produced was almost 1:1 in the presence of formate or hydrogen, but in the absence of these electron donors the ratio was about 7:6 (27).

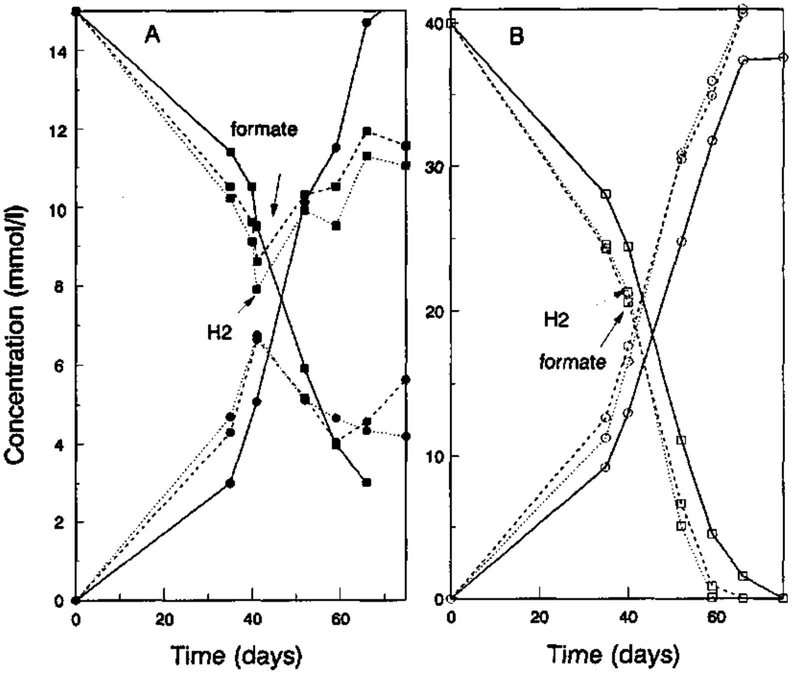

FIG. 4. Effect of $H_2$ and formate on propionate degradation (A) and fumarate degradation (B) by MPOB growing on propionate plus fumarate. Symbols: ■ propionate, ● acetate, ☐ fumarate and ○ succinate. Solid lines represent the experiment without $H_2$ and formate. The dotted lines and dashed lines represent the experiments in the presence of 162 kPa $H_2$ and 20 mM formate, respectively. The addition of $H_2$ or formate is indicated by the arrows.

## DISCUSSION

Thermodynamically both hydrogen and formate may be involved in the interspecies electron flow in syntrophic methanogenic cultures. The propionate-oxidizing bacterium (MPOB) degraded propionate in coculture with methanogens which were able to use both hydrogen and formate. However, in cocultures with methanogens which are only able to use hydrogen, propionate was not degraded, unless bacteria were present which are able to convert formate into $H_2$ + $CO_2$. These findings indicate clearly that low formate and low hydrogen concentrations are required for the syntrophic propionate oxidation by MPOB, and They give support to the hypothesis that besides hydrogen transfer formate transfer is an important process as well (3, 30). Our data do not allow to quantify the relative importance of the two processes. Boone et al (3) calculated that in a syntrophic butyrate-degrading culture formate transfer rate was 98-fold higher than the rate of hydrogen transfer.

The propionate-degradation rate in the coculture with *M. formicicum* was lower than in the coculture with *M. hungatei*, 0.8 mM/day vs. 0.4 mM/day, respectively. The $K_M$ value of the formate dehydrogenase of *M. formicicum* is 0.58 mM and that of the enzyme of M. *hungatei* is 0.22 mM, the formate threshold values of these methanogens are 26 $\mu$M and 15 $\mu$M, respectively (23). In contrast, the $K_M$ values of the hydrogenase of these two methanogens are about the same, 6 and 5 $\mu$M, and the threshold values for hydrogen are 16.6 and 17.8 nM, respectively. In syntrophic degradation the rate of conversion is determined by the rate of diffusion of hydrogen and/or formate from the acetogenic to the methanogenic bacterium. Diffusion rates of $H_2$ and formate are determined by the distance between the bacteria, the diffusion coefficients of $H_2$ and formate, and the gradients of $H_2$ and formate between the bacteria (24). Therefore, the differences in the propionate degradation rates in the cocultures can be explained best when the propionate oxidation rates are mainly determined by the concentration of formate and not by the concentration of hydrogen. Propionate is oxidized to acetate via the methylmalonyl-CoA pathway (10, 12, 16). The oxidation of succinate to fumarate coupled to bicarbonate reduction to formate ($\Delta G°' = 84.7$ kJ/mol) or coupled to proton reduction to hydrogen ($\Delta G°' = 86.2$ kJ/mol) is the energetically most difficult step of this pathway. MPOB is also able to reduce fumarate to succinate with hydrogen or formate as electron donors (27), and therefore, both hydrogen and formate will severely inhibit the succinate oxidation step during syntrophic growth on propionate. This implies that both the formate and the hydrogen concentrations have to be kept low in order to let propionate oxidation proceed. The observation that propionate can be degraded in tricultures in which a bacterium is present which is able to convert formate into hydrogen strongly suggests that hydrogen transfer can occur provided that low formate concentrations are maintained. Inhibition by formate might also explain why the shortening of the distance between MPOB and *Methanobrevibacter* by co-precipitation with FeS was without effect, and also why in tricultures consisting of this $H_2$-consuming methanogen and *Methanothrix soehngenii* propionate degradation did not occur despite the fact that both the hydrogen and the acetate concentration were kept low. At present it is not clear yet how formate is formed by MPOB. During propionate oxidation reducing equivalents are formed in three steps: succinate oxidation to fumarate, malate oxidation to oxaloacetate and pyruvate oxidation to acetyl-CoA. Cell free extracts of MPOB contained hydrogenase and formate dehydrogenase activity (21), indicating that the bacterium is able to form both hydrogen and formate. MPOB was aslo able to interconverted $H_2$ and formate. However, hydrogen was only formed when the formate concentration was above 0.9 mM and formate formation only occurred when the $H_2$ partial pressure was above 80 kPa (unpublished data). During syntrophic growth on propionate $H_2$ and formate concentrations were much lower, about 16 Pa and $< 0.1$ mM, respectively. Formate can also be formed when pyruvate oxidation is catalyzed by a pyruvate formate lyase rather than by a ferredoxin dependent pyruvate dehydrogenase (15, 28). Thus far we were unable to detect pyruvate formate lyase activity in cell extracts of MPOB.

It can be calculated that propionate oxidation can not pulled by low acetate concentrations alone. Therefore, it was not supprising that cocultures of MPOB and *M. soehngenii* did not degrade propionate. However, propionate was degraded much faster in tricultures consisting of MPOB, *M. hungatei* and *M. soehngenii* than in bicultures in which this aceticlastic methanogen was absent. The propionate degradation rates were 1.4 and 0.8 mM/day, respectively. The lowest acetate concentration that can be reached by *M. soehngenii* is about 10 $\mu$M (14). Such low acetate concentrations will be favorable for the syntrophic oxidation of propionate. It can be calculated the $\Delta G'$ value of propionate oxidation is about 17 kJ lower when the acetate concentration is 10 $\mu$M rather than 10 mM. The beneficial effect of low acetate concentrations on syntrophic degradation of fatty acids has been reported before (1, 4, 27). In addition, syntrophic conversion of acetone + $CO_2$ to 2 acetate ($\Delta G°' = -31.0$ kJ/mol) is completely driven by interspecies acetate transfer (19).

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## Chapter 3

# Butyrate degradation by *Syntrophospora bryantii* in coculture with different methanogens and in pure culture with pentenoate as electron acceptor

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## Abstract

*Syntrophospora bryantii* degraded butyrate in coculture with methanogens which can use both hydrogen and formate for growth, but not in coculture with methanogens which metabolize only hydrogen, suggesting that in suspended cultures formate may be a more important electron carrier in the syntrophic degradation of butyrate than hydrogen. Syntrophic butyrate oxidation was inhibited by the addition of 20 mM formate or the presence of 130 kPa hydrogen. In the absence of methanogens, *S. bryantii* is able to couple the oxidation of butyrate to acetate to the reduction of pentenoate to valerate. Under these conditions, up to 300 Pa hydrogen was measured in the gas phase and up to 0.3 mM formate in the liquid phase. *S. bryantii* was unable to grow syntropically with the aceticlastic methanogen *Methanothrix soehngenii*. However in triculture with *Methanospirillum hungatei* and *M. soehngenii*, *S. bryantii* degraded butyrate faster than in a biculture with only *M. hungatei*. While addition of 50 mM acetate significantly decreased the degradation rate of *S. bryantii* coculture with *M. hungatei*, showing that a low acetate concentration is advantageous. Hydrogenase and formate dehydrogenase activities were demonstrated in cell-free extracts of *S. bryantii*.

## Introduction

Butyrate is an important intermediate in the anaerobic conversion of complex organic matter under methanogenic conditions. Its oxidation may account for 8% of the total methane formation in bioreactors (Mah et al. 1990). In methanogenic habitats, butyrate oxidation is accomplished by syntrophic consortia of acetogenic and methanogenic bacteria (Table 1). Acetogenic bacteria oxidize butyrate to two acetate. This reaction is highly endergonic, and therefore methanogens are needed to pull butyrate oxidation either by the removal of hydrogen or by the removal of formate (McInerney et al. 1981a; Henson and Smith 1985; Stieb and Schink 1985; Boone et al. 1989). The acetate which is formed during butyrate oxidation is cleaved by aceticlastic methanogens. Table 1 shows how the Gibbs free energy change of the reactions involved in butyrate oxidation depends on the concentration of formate, hydrogen and acetate. Threshold values of methanogens for hydrogen, formate and acetate are 2.5-100 Pa, 15-26 $\mu$M and 7-200 $\mu$M (Seitz et al. 1988; Schauer et al. 1982; Jetten et al. 1990).

Up to now it is unclear whether during syntrophic degradation hydrogen transfer or formate transfer is the most important mechanism by which reducing equivalents are shuttled from the acetogens to the methanogens. The occurrence of hydrogen transfer has been demonstrated in butyrate-degrading cocultures in which methanogens were present which only catabolize hydrogen but not formate (McInerney et al. 1981a,b; Henson and Smith 1985). A possible role for formate transfer was put forward by Thiele and Zeikus (1988). Boone et al (1989) calculated by using diffusion models that the rate of methane formation in a coculture of *Syntrophomonas wolfei* and *Methanobacterium formicicum* could not be explained by hydrogen transfer, but it could be explained by formate transfer.

Butyrate-oxidizers *Syntrophomonas wolfei* (McInerney et al. 1981a) and *Syntrophospora (Clostridium) bryantii* (McInerney et al. 1979; Stieb and Schink 1985; Zhao et al. 1989) degrade butyrate and other short-chain fatty acids in syntrophic association with methanogens or sulfate-reducing bacteria. They can also grow in pure culture on crotonate and other short-chain unsaturated fatty acids (Beaty and McInerney 1987; Zhao et al. 1989; Amos et al. 1990). This fermentative property was used to obtain pure cultures of the two bacteria. In this study cocultures of *S. bryantii* with different methanogens were constructed to investigate the role of formate and hydrogen transfer. In addition, the formation of $H_2$ and formate during butyrate oxidation and the presence of hydrogenase and formate dehydrogenase were investigated.

**Table 1.** Reactions possibly involved in the syntrophic degradation of butyrate, and the Gibbs free energy changes of the reactions[a]

| Reactions | $\Delta G^{0'}$ | $\Delta G'_1$ | $\Delta G'_2$ | $\Delta G'_3$ |
|---|---|---|---|---|
| | (kJ/mol) | | | |
| **butyrate degradation:** | | | | |
| 1. $CH_3CH_2CH_2COO^- + 2H_2O \dashrightarrow 2CH_3COO^- + H^+ + 2H_2$ | 48.1 | -5.3 | | -11.7 |
| 2. $CH_3CH_2CH_2COO^- + 2HCO_3^- \dashrightarrow 2CH_3COO^- + H^+ + 2HCOO^-$ | 45.5 | | -10.5 | -14.3 |
| **Methanogenesis:** | | | | |
| 3. $4H_2 + CO_2 \dashrightarrow CH_4 + 2H_2O$ | -130.7 | -35.6 | | |
| 4. $4HCOO^- + 4H^+ \dashrightarrow CH_4 + 2H_2O + 3CO_2$ | -144.5 | | -50.7 | |
| 5. $CH_3COO^- + H^+ \dashrightarrow CH_4 + CO_2$ | -36 | | | -6.1 |

a: $\Delta G'_1$, $\Delta G'_2$ and $\Delta G'_3$ represent the calculated Gibbs free energy changes when the hydrogen partial pressure is 2.5 Pa, the formate concentration is 15 $\mu$M or the acetate concentration is 7 $\mu$M, respectively.

## Materials and methods

*Organisms.* Unless stated otherwise all the bacterial cultures were obtained from the German culture collection of microorganisms (DSM; Braunschweig, Germany). *Syntrophospora bryantii* was isolated by us from a biculture with *Methanospirillum hungatei* (DSM 3014B) using crotonate as the sole carbon and energy source (Zhao et al. 1989). The following hydrogenotrophic methanogens were used: *Methanospirillum hungatei* DSM 864 (Ferry et al. 1974), *Methanobacterium formicicum* DSM 1535 (Bryant et al. 1987), *Methanobrevibacter arboriphilus* DSM 1125 (Zeikus et al. 1975) and *M. arboriphilus* DSM 744 (Zehnder et al. 1977). The aceticlastic methanogen, *Methanothrix soehngenii* (Huser et al. 1982) was from our own culture collection.

*Media and culture conditions.* A basal bicarbonate-buffered mineral medium as described by Houwen et al. (1987) supplemented with 10 g/l of NaCl, 0.2 g/l yeast extract and 0.2 g/l biotrypticase was used for all the bacterial strains. The trace minerals $Na_2SeO_3$ and $Na_2WO_4$ were omitted from the media. Media for the growth of *M. soehngenii* contained 5 g/l sodium acetate as carbon source, and a gas phase of 162 kPa $N_2/CO_2$ (80/20) was applied above the media. All other methanogens were grown on 162 kPa $H_2/CO_2$ (80/20). *S. bryantii* was routinely grown in media containing 1.72 g/l crotonate under a gas atmosphere of 162 kPa $N_2/CO_2$ (80/20). All the bacteria were cultured at 37°C in 120-ml serum vials (Aluglas Verenigde Bedrijven B.V., Amersfoort, The Netherlands) containing 50 ml medium. The vials were sealed with butylrubber stoppers and aluminium caps (Rubber B.V., Hilversum, The Netherlands).

*Butyrate oxidation by suspended bi- and tricultures.* Methanogens were pregrown in the media described above till the end of exponential phase. *S. bryantii* was grown on crotonate till all the crotonate was degraded. *M. soehngenii* was grown on acetate till the methane formation from acetate had ceased. The suspended bicultures were organized by adding 10 ml crotonate-grown *S. bryantii* cells to 40 ml cells of the hydrogenotrophic methanogens. For the suspended tricultures, 10 ml crotonate-grown *S. bryantii* was added to a mixture of 20 ml the hydrogenotrophic methanogens and 20 ml of the aceticlastic methanogen. The gas phase was changed to 162 kPa $N_2/CO_2$ (80/20), and butyrate was added from 1M stock solutions to give a final concentration of 20 mM. All these procedures were done aseptically using syringes and heat-sterilized cotton filters. Vials were incubated in the dark, and after various periods of time liquid and gas samples were withdrawn for analysis. The maximum butyrate degradation rates were estimated from the highest slope of the butyrate degradation curve. Rates were experessed per miligram cell protein. Total cell protein of the cultures was determined at the end of the incubation by the Lowry method (Lowry et al. 1951).

*Substrate conversion by pure culture of S. bryantii.* Crotonate and pentenoate degradation rates were determined by adding 2 ml crotonate-grown cells to 50 ml fresh medium containing 20 mM crotonate and pentenoate respectively. Butyrate degradation rates were measured by using crotonate pregrown dense cultures to which 20 mM pentenoate plus 10 mM butyrate were added. Sampling and sample preparation for analysis were done as described above.

*Enzyme assays.* Crotonate-grown cells were harvested by centrifugation at 10,000 rpm for 15 min and broken by passing through a French pressure cell at 40 MPa. The cell-free extract for enzyme assays was obtained by centrifugation of the crude extract at 12,000 rpm at 4°C for 20 min. Methylviologen-coupled hydrogenase and methylviologen-coupled formate dehydrogenase activities were measured at pH 8.0 as described by Lamed and Zeikus (1980) and Leonhardt and Andreesen (1977), respectively. One unit of enzyme activity was expressed by the amount of enzyme catalyzing 1 $\mu$mol of $H_2$ or formate per min.

*Analytical methods.* $H_2$ and methane were measured gas chromatographically as described before (Houwen et al. 1988). Formate, acetate, propionate, butyrate, pentenoate, crotonate and valerate were measured by HPLC (Dong et al. 1991).

## Results

### *Butyrate degradation in constructed cocultures.*

The ability of *S. bryantii* to grow in pure culture with crotonate offered us the possibility to study the butyrate degradation kinetics in defined co- and tricultures with different methanogens. Fig. 1 shows that *S. bryantii* degraded butyrate in the presence of *M. formicicum* (A) or *M. hungatei* (B). Cocultures with two *M. arboriphilus* strains (C) and with

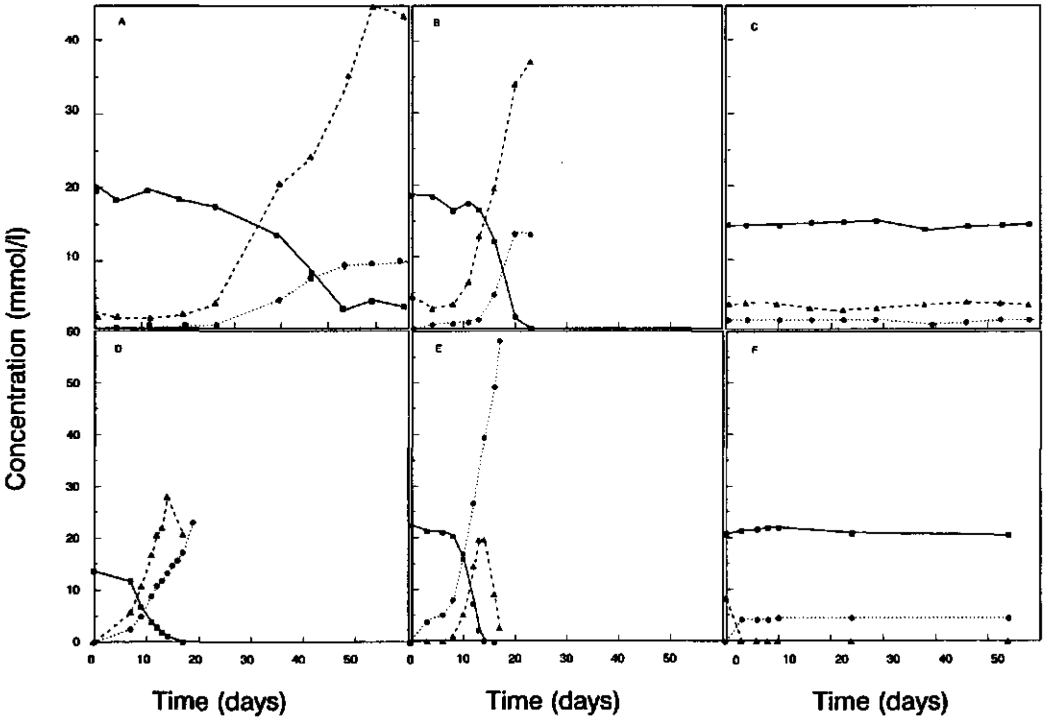

**Fig. 1.** Butyrate (■) degradation and acetate (▲), methane (●) production by *S. bryantii* in cocultures with *M. formicicum* (A), *M. hungatei* (B), *M. arboriphilus* DSM 1125 (C) and *M. soehngenii* (F) and in triculture with *M. formicicum* and *M. soehngenii* (D), and in triculture with *M. hungatei* and *M. soehngenii* (E).

*M. soehngenii* (F) were tested as well. With none of these strains butyrate oxidation occurred. The time course of butyrate oxidation differed considerably in the different co- and tricultures. In the coculture of *M. formicicum* the degradation was much slower than in coculture with *M. hungatei* (Fig 1A and 1B). The estimated degradation rates were about 0.9 $\mu$mol.h$^{-1}$.mg$^{-1}$ protein and 2.5 $\mu$mol.h$^{-1}$.mg$^{-1}$ protein, respectively. In tricultures in which the aceticlastic *M. soehngenii* was also present, the degradation rates were always higher than in the corresponding bicultures. The butyrate degradation rate in a triculture with *M. formicicum* (Fig. 1D) was about 2.2 $\mu$mol.h$^{-1}$.mg$^{-1}$ protein, while in a triculture with *M. hungatei* (Fig. 1E) the degradation rate was about 3.2 $\mu$mol.h$^{-1}$.mg$^{-1}$ protein. The degradation of butyrate by a biculture of *S. bryantii* and *M. hungatei* was totally inhibited by the addition of either 130 kPa $H_2$ (Fig. 2A) or 20 mM formate (Fig. 2B), but the $CH_4$ formation continued. Butyrate degradation by this coculture was not strongly affected by moderately high

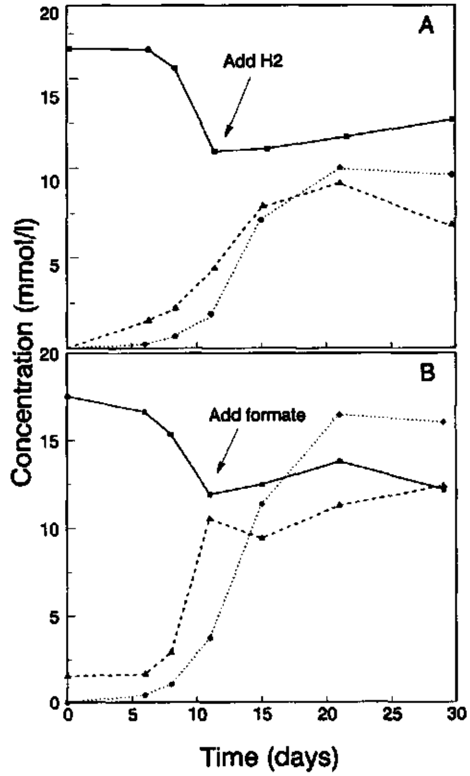

**Fig. 2.** Inhibition of butyrate degradation by 130 kPa hydrogen (A) and 20 mM formate (B) in coculture of *S. bryantii* with *M. hungatei*. Symbols: ■, butyrate; ▲, acetate; ●, methane.

acetate concentration (Fig. 3). After the addition of 50 mM acetate the butyrate degradation rate decreased about 60%, and the degradation stopped when the acetate concentration had increased till about 60 mM.

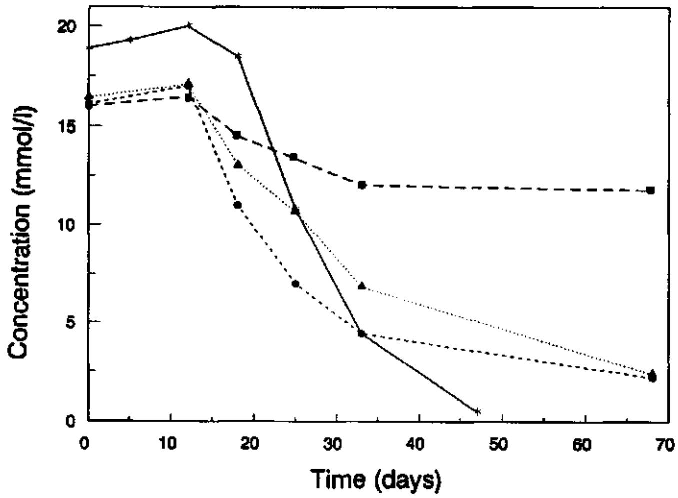

**Fig. 3.** Effect of acetate on butyrate degradation by coculture of *S. bryantii* with *M. hungatei*. Symbols: *, control; ●, 30 mM acetate added; ▲, 40 mM acetate added; and ■, 50 mM acetate added.

*Growth of S.bryantii in pure culture.*

*S. bryantii* in the absence of methanogens showed fast fermentative growth on crotonate and pentenoate, the maximum specific growth rates were about 0.4 day$^{-1}$. The maximum specific growth rate during syntrophic growth on butyrate was about 0.11 day$^{-1}$. Crotonate was fermented to acetate and butyrate, while pentenoate was fermented to acetate, propionate and valerate (Table 2 and 3, Fig. 4A). In the presence of hydrogen or formate, the fermentation of crotonate was shifted to the formation of more butyrate and less acetate. In the presence

**Table 2.** Degradation of pentenoate by *S. bryantii* in the presence and absence of butyrate and *M. hungatei*

| Substrates | Substrate degraded(mmol/l) | | Products formed(mmol/l) | | | |
|---|---|---|---|---|---|---|
| | pentenoate | butyrate | valerate | acetate | propionate | $CH_4$ |
| pentenoate | 8.7±0.5 | 0 | 3.3±0.1 | 4.8±0.1 | 5.2±0.5 | 0 |
| pentenoate+ butyrate | 19.4±1.5 | 6±0.8 | 17.8±0.7 | 17.5±0.1 | 5.3±0.2 | 0 |
| pentenoate+ butyrate + *M. hungatei* | 17±1.6 | 7.2±0.6 | 13.1±0.1 | 22.6±1.7 | 8.1±0.2 | 2.7±0.2 |
| butyrate+ *M. hungatei* | 0 | 16.5±0.9 | 0 | 33.3±0.4 | 0 | 13.9±0.1 |

**Table 3.** Effect of hydrogen and formate on crotonate conversion by *S. bryantii*

| substrates (mM) | crotonate degraded (mM) | butyrate produced (mM) | acetate produced (mM) | cell protein (mg/l) | degrading velocity (mM/h) |
|---|---|---|---|---|---|
| crotonate | 21.7±0.2 | 8.7±0.5 | 19.9±1.1 | 63±5 | 0.2 |
| crotonate + 20mM formate | 19.2±0.4 | 15.0±0.2 | 15.0±0.2 | 56±5 | 0.1 |
| crotonate + 0.6atm.$H_2$ | 18.5±0. 3 | 14.6±0.2 | 16.3±0.2 | 53±5 | 0.2 |

of hydrogen or formate the fermentation of pentenoate was shifted to the formation of more valerate and less propionate and acetate (data not shown). The presence of these electron donors led to a somewhat lower specific growth rate on crotonate and lower molar cell yields (Table 3). When *S. bryantii* was grown on pentenoate plus butyrate, these compounds were degraded simutaneously (Fig. 4B). In the presence of butyrate, more valerate and less propionate was formed compared with the amounts which had been formed after growth on pentenoate alone (table 2). This indicates that pentenoate can act as an electron acceptor during butyrate oxidation, and is thus able to replace methanogens. Butyrate degradation stopped when the pentenoate and butyrate had reached concentrations of about 3.5 mM, but pentenoate was degraded further (Fig. 4B). Remarkably, about 0.3 mM formate was observed at day 4 of the incubation and this concentration remained constant thereafter. In this culture, the hydrogen concentration increased till about 300 Pa. When a coculture of *S. bryantii* and *M. hungatei* was grown on pentenoate plus butyrate, methane was formed as well (Table 2). It can be calculated that

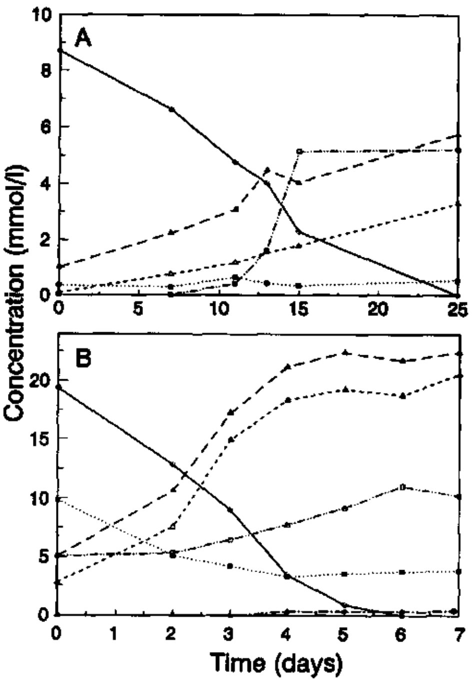

**Fig. 4.** Degradation of pentenoate by *S. bryantii* in the absence (A) and presence (B) of butyrate, and butyrate degradation with pentenoate as electron acceptor (B) by crotonate-pregrown culture of *S. bryantii*. Symbols: ○, pentenoate; Δ, valerate; ■, butyrate; ▲, acetate; □ propionate; ●, formate.

under these conditions about 75 % of the electrons produced in the oxidation of butyrate to acetate were transferred to the methanogen, whereas the remainder was used to reduce pentenoate to valerate.

*Hydrogenase and formate dehydrogenase activities.*

The specific activity of methylviologen-coupled hydrogenase in cell-free extract of crotonate-grown cells of *S. bryantii* was 59.4 $\mu$mol.min$^{-1}$.mg$^{-1}$ protein. The maximum specific activity of formate dehydrogenase was 0.045 $\mu$mol.min$^{-1}$.mg$^{-1}$ protein. For the two enzymes an apparent $K_M$ value of 0.21 mM $H_2$ and 0.22 mM formate was determined in cell extracts.

## Discussion

*S. bryantii* can grow on crotonate in pure culture (Zhao et al. 1989). This property allowed us to investigate the role of hydrogen and formate transfer in reconstructed methanogenic cocultures. Butyrate was degraded only in cocultures with methanogens which can use both $H_2$ and formate, like *M. hungatei* and *M. formicicum*, but not in cocultures with methanogens which can use only $H_2$, like *M. arboriphilus* nor which can only use acetate, like *M. soehngenii*. This suggests that formate plays an important role in the syntrophic degradation of butyrate. These findings give support to the hypothesis that in suspended cultures formate transfer is more important than hydrogen transfer. Dwyer et al. (1988) pointed out that the rate of hydrogen consumption during syntrophic degradation is largely determined by the affinity constant ($K_M$) of the $H_2$ utilizing bacteria for hydrogen. Comparing the $K_M$ values for $H_2$ and formate of *M. hungatei* and *M. formicicum*, it is obvious that the $K_M$ values for $H_2$ are in the same range (5 and 6 $\mu$M respectively), while the $K_M$ values for formate are clearly different, 0.22 mM for *M. hungatei* and 0.58 mM for *M. formicicum* (Table 4). If formate transfer is important, these differences may explain why cocultures with *M. hungatei* had higher butyrate degrading rates than the cocultures with *M. formicicum*.

Both hydrogen and formate inhibit butyrate oxidation by *S. bryantii*. In addition, the organism is able to use formate and hydrogen as electron acceptors during fermentative growth on crotonate, and the bacterium contains both hydrogenase and formate dehydrogenase activity. Direct evidence for the formation of formate and hydrogen by *S. bryantii* was obtained during growth in pure culture on mixtures of butyrate and pentenoate. *S. bryantii* ferments pentenoate in a similar fashion as described for *Syntrophomonas wolfei* (Amos et al. 1990); one molecule of pentenoate is oxidized to one molecule of propionate and one molecule of acetate, and the reducing equivalents are used to reduce another molecule of pentenoate to valerate. When *S. bryantii* is cultivated on pentenoate plus butyrate, it oxidizes one molecule of butyrate into two molecule of acetate and reducing equivalents. These reducing equivalents are used to reduce pentenoate to valerate. Under these conditions, up to 300 Pa hydrogen was detected in the gas phase, while up to 0.3 mM formate was measured in the liquid phase. In the absence of butyrate, the levels of hydrogen and formate were lower than 30 Pa and $<0.1$ mM, respectively. These observations show that *S. bryantii* is able to form $H_2$ and formate during butyrate conversion. It is known that the lowest $H_2$ and formate concentrations that can be maintained by *M. hungatei* are 3 Pa

**Table 4.** Butyrate degradation rates by *S. bryantii* in different methanogenic cocultures and tricultures, and in pure culture of with pentenoate as electron acceptor

| cultures | specific rates ($\mu$mol.$h^{-1}$.$mg^{-1}$ protein) | $K_M$ of $H_2$-consumer | |
|---|---|---|---|
| | | $H_2$($\mu$M) | formate($\mu$M) |
| *S.bryantii*<br>+penteonate | 1.1 | | |
| *S.bryantii*<br>+*M.hungatei* | 2.5 | 5.0[b] | 0.22[a] |
| *S.bryantii*<br>+*M.hungatei*<br>+*M.soehngenii* | 3.2 | 5.0 | 0.22 |
| *S.bryantii*<br>+*M.formicicum* | 0.9 | 6[a] | 0.58[a] |
| *S.bryantii*<br>+*M.formicicum*<br>+*M.soehngenii* | 2.2 | 6[a] | 0.58[a] |
| *S.bryantii*<br>+*M.arboriphilus* | 0 | 6[c] | N.G.[d] |
| *S.bryantii*<br>+*M.soehngenii* | 0 | N.G | N.G. |

a: Schauer et al. (1982)
b: Robinson and Tiedje (1984)
c: Kristjansson et al. (1982)
d: N.G., no growth of the methanogen

(Cord-Ruwisch et al. 1988) and 26 $\mu$M (Schauer et al. 1982) respectively. Thus the concentration difference of formate between *S. bryantii* and *M. hungatei* is about 150 times higher than the concentration difference of $H_2$ between the two species. The diffusion coefficients of $H_2$ and formate in water are 0.0045 and 0.0015 $mm^2.s^{-1}$, respectively (Boone et al. 1989). By using a simple Ficks diffusion equation (Schink and Thauer 1988), it can be calculated that the flux of formate can be about 50 times as high as the flux of hydrogen, indicating that formate transfer is more important than hydrogen transfer in our coculture.

Enzyme measurements by Wofford et al. (1986) showed that *S. wolfei* oxidized butyrate via the crotonyl-CoA pathway. A similar pathway is present in *S. bryantii* (Schink 1992). Two oxidation steps occur in this pathway, the butyryl-CoA oxidation to crotonyl-CoA and the 3-hydroxybutyryl-CoA oxidation to acetoacetyl-CoA. Both reactions are thermodynamically unfavorable when coupled to hydrogen and formate formation. The Gibbs free energy change of the oxidation of butyryl-CoA to crotonyl-CoA and 3-hydroxybutyryl-CoA to acetoacetyl-CoA are +75.2 and +29.3 kJ/mol when coupled to hydrogen formation, and +73.9 and 28 kJ/mol when coupled to formate formation (Thauer et al. 1977). Because *S. bryantii* is able to produce hydrogen and formate, these calculations show that both hydrogen and formate will inhibit butyrate oxidation, irrespectively whether hydrogen or formate transfer is most important.

Although acetate is one of products in butyrate oxidation, cocultivation with the aceticlastic methanogen *M. soehngenii* is not sufficient to allow butyrate degradation by *S. bryantii*. However, methanogenic tricultures of *S. bryantii*, *M. soehngenii* and *M. hungatei* or *M. formicicum* had a higher degrading rate than the cocultures without *M. soehngenii*. This indicates that a low acetate concentration is an additional requirement for achieving high butyrate degradation rates in methanogenic habitats. Similar observations have been found by Ahring and Westermann (1987, 1988) and Beaty and McInerney (1989).

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## Chapter 4

# Evidence for $H_2$ and formate formation during syntrophic butyrate and propionate degradation

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Anaerobe (Submitted)

## Abstract

Both $H_2$ and formate were formed during butyrate oxidation by *Syntrophospora bryantii* with pentenoate as electron acceptor and during propionate oxidation by a mesophilic propionate oxidizing bacterium (MPOB) with fumarate as electron acceptor. $H_2$ and formate levels were affected by the bicarbonate concentration. *S. bryantii* and MPOB were also able to interconvert formate and $H_2$ + $HCO_3^-$; the apparent $K_M$ values for formate were of 2.9 mM and 1.8 mM, respectively. The conversion of $H_2$ + $HCO_3^-$ to formate occurred only when the $H_2$ partial pressure was above 80 KPa. This interconversion seems rather unimportant under conditions prevailing during syntrophic propionate and butyrate oxidation.

*Key words*: $H_2$, formate, syntrophic degradation, butyrate oxidation, propionate oxidation, interspecies electron transfer, methanogenesis

## Introduction

Butyrate and propionate are two important intermediates in the mineralization of organic matter under methanogenic conditions. These fatty acids are degraded by syntrophic consortia of acetogenic bacteria and methanogenic bacteria [1, 2]. Complete mineralization of butyrate and propionate into methane and $CO_2$ requires three different types of bacteria. Acetogenic bacteria oxidize butyrate and propionate into acetate and $H_2$/formate (Table 1, equation 1, 2, 5 and 6). These products are further converted to methane by $H_2$/formate-utilizing and aceticlastic methanogens (Table 1, equation 9, 10 and 11), respectively. Because of unfavorable energetics of propionate and butyrate oxidation, the $H_2$ and/or formate levels have to be kept extremely low. At low $H_2$ and formate concentrations the $\Delta G'$ values of butyrate and propionate oxidation become negative. Interspecies $H_2$ and formate transfer are proposed as possible mechanisms for the syntrophic degradation by consortia [3-5]. Although both $H_2$ and formate were detected in the methanogenic cocultures when methanogenesis was inhibited [4, 6], it is difficult to conceive whether $H_2$ or formate is more important. This in particular because methanogens are able to interconvert $H_2/CO_2$ and formate [7, 8]. Up to now, direct evidence is lacking that butyrate-degrading and propionate-degrading acetogens can form both $H_2$ and formate.

*Syntrophomonas wolfei* [9] is the first described acetogen which can oxidize butyrate. Since then several butyrate-oxidizing bacteria were isolated and characterized, such as *Syntrophospora bryantii* [10, 11], *Syntrophomonas sapovorans* [12] and strain SF-1 [13]. It was demonstrated that *S. wolfei* and *S. bryantii* oxidized butyrate by $\beta$-oxidation via the crotonyl-CoA pathway [14, 1]. Reducing equivalents are released during the oxidations of butyryl-CoA to crotonyl-CoA and of 3-hydroxybutyryl-CoA to acetoacetyl-CoA. Boone and Bryant [15] described the first propionate oxidizer *Syntrophobacter wolinii* growing in a defined coculture with *Desulfovibrio* G11. Recently we enriched a mesophilic propionate oxidizing-bacterium (MPOB) in coculture with *Methanospirillum* strain, and obtained a methanogen-free culture by growing it on fumarate [16]. Enzyme assays [17, 18] and experiments of $^{13}C$ and $^{14}C$ labelled propionate [19-22] have indicated that the methylmalonyl-CoA pathway is involved in syntrophic propionate oxidation. Reducing equivalents are

Table 1. Reactions involved in the syntrophic degradation of butyrate and propionate

| Reactions | | $\Delta G^{o\prime}$(kJ/reaction)[a] |
|---|---|---|
| 1. Butyrate$^-$ + 2 $H_2O$ | ---> 2 Acetate$^-$ + $H^+$ + 2 $H_2$ | 48.1 |
| 2. Butyrate$^-$ + 2 $HCO_3^-$ | ---> 2 Acetate$^-$ + $H^+$ + 2 $HCOO^-$ | 45.5 |
| 3. Pentenoate$^-$ + $H_2$ | ---> Valerate$^-$ | -75.2[b] |
| 4. Pentenoate$^-$ + $HCOO^-$ +$H_2O$ | ---> Valerate$^-$ + $HCO_3^-$ | -73.8[b] |
| 5. Propionate$^-$ + 3 $H_2O$ | ---> Acetate$^-$ + $HCO_3^-$ + 3 $H_2$ + $H^+$ | 76 |
| 6. Propionate$^-$ + 2 $HCO_3^-$ | ---> Acetate$^-$ + 3$HCOO^-$ + $H^+$ | 72 |
| 7. Fumarate$^{2-}$ + $H_2$ | ---> Succinate$^{2-}$ | -86.2 |
| 8. Fumarate$^{2-}$ + $HCOO^-$ + $H_2O$ | ---> Succinate$^{2-}$ + $HCO_3^-$ | -84.9 |
| 9. 4 $H_2$ + $HCO_3^-$ + $H^+$ | ---> $CH_4$ + 2 $H_2O$ | -135.6 |
| 10. 4 $HCOO^-$ + $H_2O$ + $H^+$ | ---> $CH_4$ + 3 $HCO_3^-$ | -130 |
| 11. $CH_3COO^-$ + $H_2O$ | ---> $CH_4$ + $HCO_3^-$ | -31 |
| 12. $H_2$ + $HCO_3^-$ | ---> $HCOO^-$ + $H_2O$ | -1.3 |

a, $\Delta G^{o\prime}$ values were obtained from Thauer et al. [30].

b, It is assumed that the $\Delta G^{o\prime}$ values of the reduction of pentenoate to valerate are similar to those of the reduction of crotonate to butyrate.

formed in the oxidations of succinate to fumarate, malate to oxaloacetate and pyruvate to acetate, respectively.

In our laboratory, it was found that *S. bryantii* degraded butyrate in pure culture in the presence of pentenoate as electron acceptor [6], and a mesophilic propionate-degrading bacterium (MPOB) [16] degraded propionate with fumarate as electron acceptor. By using limiting amounts of electron acceptors we describe here that both $H_2$ and formate are produced by *S. bryantii* and MPOB. The ratio at which formate and hydrogen are formed depends on the bicarbonate concentration.

## Materials and methods

*Organisms and cultivation*:

*Syntrophospora bryantii* (DSM 3014B) in coculture with *Methanospirillum hungatei* was obtained from the German Collection of Microorganisms (Braunschweig, Germany). *S. bryantii* was isolated from the coculture with crotonate as substrate as described by Zhao et al. [11] in a medium with a composition as described previously [21]. Subcultivation was done with 2.2 g/l sodium crotonate under a gas phase of 162 KPa $N_2/CO_2$. The mesophilic propionate-oxidizing bacterium (MPOB) was described by Stams et al. [16]. Routinely, the bacteria were grown in a mineral medium described previously [21] containing sodium fumarate (6.5 g/l) under a gas phase of 162 KPa $N_2/CO_2$.

### *$H_2$ and formate interconversion*:

One liter of crotonate-grown *S. bryantii* cells and fumarate-grown MPOB cells were harvested by anaerobic centrifugation at 10,000 rpm for 15 minutes. The pellets were resuspended in 50 ml mineral medium without substrate. The suspension was dispensed in portions of 1 ml in 9-ml serum bottles. The conversion of $H_2$ + $HCO_3^-$ to formate was started by adding different ratios of $H_2$ to the serum bottles, and the $H_2$ decrease was followed in time. The conversion of formate into $H_2$ + $HCO_3^-$ was started by adding different concentrations of formate, and the $H_2$ increase was followed in time.

### *Effect of the bicarbonate concentration on $H_2$ and formate formation*:

*S. bryantii* was grown in the same medium as above but with different concentrations of bicarbonate. For the tests of without bicarbonate, 50 mM phosphate buffer (pH 7) was used instead. 5 mM pentenoate and 20 mM butyrate were added as substrates. $H_2$ and formate were measured when the pentenoate was consumed completely. A similar experiment was done with MPOB cells with 5 mM fumarate and 20 mM propionate as substrates.

### *Analytical methods*:

$H_2$ was determined gas chromatographically [21]. Formate, acetate, propionate, butyrate, pentenoate and valerate were measured by HPLC [23]. Formate was also measured by Lang

& Lang method [24]; where necessary the culture liquid was concentrated on Büchi RE121 Rotavapor at 470 Pa. at 50°C. Whole cell protein was measured by the Lowry method [25].

## Results

*$H_2$ and formate production by butyrate- and propionate-oxidizing acetogens*:

Theoretically, both $H_2$ and formate can be produced during syntrophic oxidation of propionate and butyrate (table 1). The maximal concentrations of $H_2$ and formate that was reached during butyrate oxidation by *S. bryantii* and during propionate oxidation by MPOB are listed in table 2 and 3, respectively. In the presence of 17 mM butyrate plus 4 mM pentenoate, *S. bryantii* degraded 2.1 mM butyrate. Up to 280 $\mu$M formate and 171 Pa $H_2$ accumulated in the liquid and in the head space, respectively (Table 2). When the propionate-oxidizing bacterium MPOB was grown on 14 mM propionate plus 4 mM fumarate, 1.6 mM propionate was degraded with the available fumarate. In these cultures 24 $\mu$M formate and 6.8 Pa $H_2$ were measured in the liquid and in the head space, respectively (table 3). These results show that butyrate- and propionate-oxidizing bacteria were indeed able to form both $H_2$ and formate.

Table 2. $H_2$ and formate formation during butyrate degradation by *S. bryantii* with pentenoate as electron acceptor

| days | $H_2$ (Pa) | formate ($\mu$M) | pentenoate (mM) | valerate (mM) | butyrate (mM) | acetate (mM) |
|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 4.1 | 0 | 17.3 | 0.6 |
| 7 | 171 | 280 | 0 | 4.7 | 15.2 | 5.2 |

Table 3. $H_2$ and formate formation during propionate degradation by MPOB using fumarate as electron acceptor

| days | $H_2$ (Pa) | formate ($\mu$M) | fumarate (mM) | succinate (mM) | propionate (mM) | acetate (mM) |
|---|---|---|---|---|---|---|
| 0 | 0 | n.d | 4.3 | 1.4 | 13.8 | 0 |
| 7 | 6.8 | 24 | 0 | 3.3 | 12.2 | 1.6 |

*Effect of the bicarbonate concentration on $H_2$ and formate formation*:

Because both $H_2$ and formate were produced, the effect of bicarbonate on the relative $H_2$ and formate formation by *S. bryantii* was investigated. Fig.1 shows that the $H_2$ and formate production was dependent on the concentration of bicarbonate. The formate concentration increased when the bicarbonate concentration was increased, while the $H_2$ level decreased slightly.

The effect of bicarbonate on $H_2$ and formate production during propionate oxidation by MPOB was determined as well. $H_2$ accumulated to 44 Pa when the MPOB was grown on 20 mM propionate plus 5 mM fumarate in the absence of bicarbonate, whereas the formate concentration was below the detection limit (< 10 μM). However, at a bicarbonate concentration of 50 mM about 24 μM formate was formed while the $H_2$ partial pressure amounted only till 6.8 Pa.

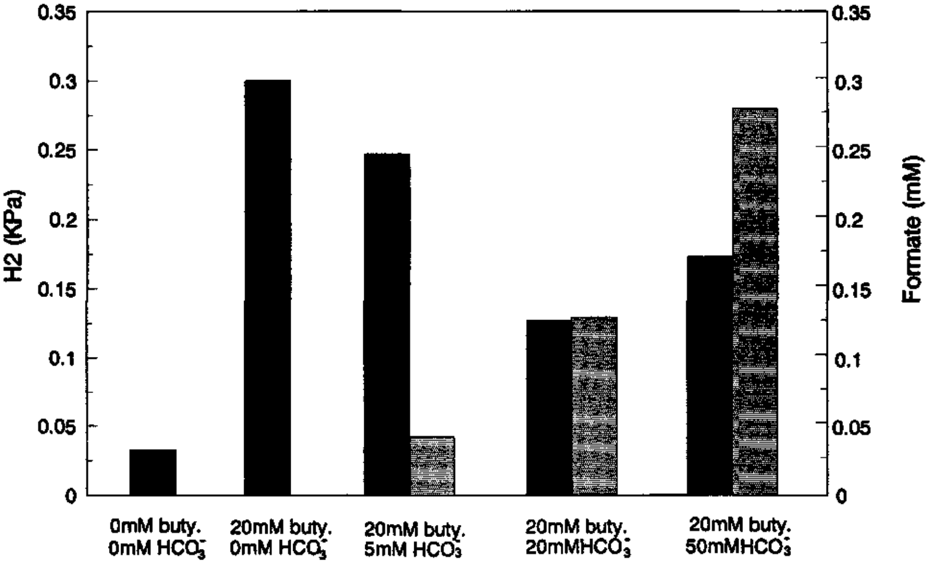

Fig.1. Hydrogen (solid bar) and formate (dotted bar) production during butyrate oxidation by *S. bryantii* at different $HCO_3^-$ concentration with limiting amount of pentenoate as electron acceptor.

*$H_2$ and formate interconversion by S. bryantii and MPOB*:

As the midpoint redox potentials of $H^+/H_2$ and $HCO_3^-$/formate are in the same range (-414 mV and -432 mV, respectively), an interconversion between formate and $H_2/HCO_3^-$ can be expected. Fig.2A and 2B shows that *S. bryantii* and MPOB are indeed able to convert formate into $H_2$ + $HCO_3^-$. The maximum rate of formate conversion by *S. bryantii* was 3.8 nmol.min$^{-1}$.mg$^{-1}$ cell protein, the $K_M$ value for formate was 2.9 mM, and the lowest concentration of formate that could be reached was about 0.5 mM (table 4). The maximum rate of formate conversion by MPOB was 4.9 nmol.min$^{-1}$.mg$^{-1}$ cell protein, the $K_M$ value for formate was 1.8 mM, and the lowest concentration of formate that could be reached was about 0.9 mM (table 4). $H_2$ + $HCO_3^-$ was also converted into formate by the two bacteria. However, formate formation was detected only when the $H_2$ partial pressure was above 80 KPa (results not shown).

Table 4. Conversion of formate to $H_2$ by *S. bryantii* and MPOB

| | $K_M$(mM) | Vmax($\mu$M min$^{-1}$.mg$^{-1}$) | lowest concentration(mM) |
|---|---|---|---|
| *S. bryantii* | 2.9 | $3.8*10^{-3}$ | 0.5 |
| MPOB | 1.8 | $4.9*10^{-3}$ | 0.9 |

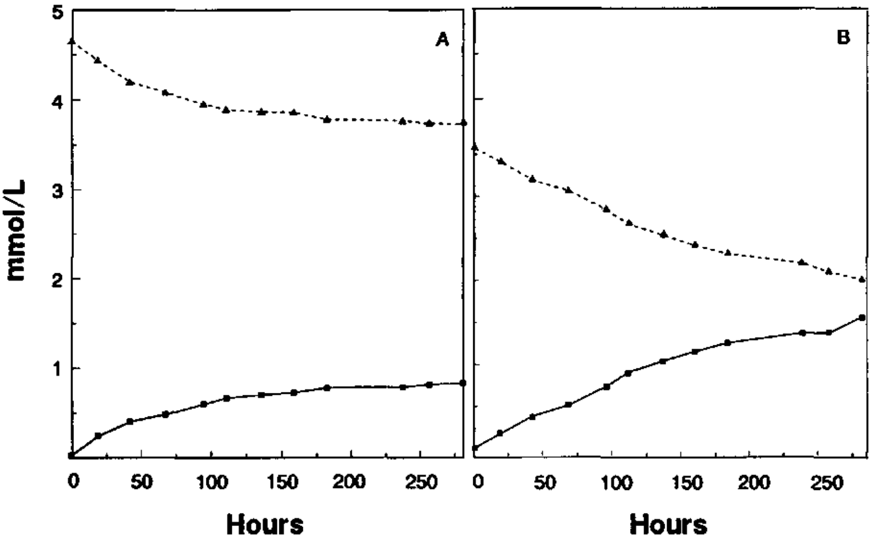

Fig.2. The conversions of formate (▲) to hydrogen (■) by dense cell suspensions of *S. bryantii* (A) and MPOB (B).

## Discussion

Theoretically, both hydrogen and formate could be produced during syntrophic butyrate and propionate oxidation. These two compounds indeed were produced by *S. bryantii* and MPOB during butyrate and propionate oxidation, respectively. The $H_2$ and formate concentrations amounted 170 Pa and 280 $\mu$M for *S. bryantii* and 7 Pa and 24 $\mu$M for MPOB. By using the fatty acids concentrations listed in table 2 and 3, a $HCO_3^-$ concentration of 38 mM and the Gibbs free energy values from table 1 the maximal hydrogen and formate concentrations that could theoretically be reached can be calculated. 205 Pa $H_2$ and 130 $\mu$M formate for *S. bryantii* and 10 Pa $H_2$ and 4.3 $\mu$M formate for MPOB can be deduced. This shows that the measured values are in the same range as what can be expected from thermodynamical considerations.

By using the highest concentrations of formate and $H_2$ that can be measured with the two bacteria and the lowest formate and $H_2$ concentrations that can be reached by methanogens, the fluxes of formate and $H_2$ between consortia in the syntrophic culture can be calculated using the Fick's diffusion equation [26]. In these calculations diffusion constants of $H_2$ and formate in water of $4.5*10^{-5}$ and $1.5*10^{-5}$ $cm^2.sec^{-1}$., respectively, were used. The threshold values for $H_2$ and formate of *M. hungatei* are 2 Pa and 15 μM, respectively [27, 28]. Table 5 shows that the calculated $H_2$ flux in the coculture of MPOB with *M. hungatei* is too low to account for the methane formation rate which had been measured [29]. However, the formate flux is sufficiently high to account for the $CH_4$ formation rate. In the coculture of *S. bryantii* with *M. hungatei* both the calculated $H_2$ flux and formate flux are much higher than needed to explain the measured $CH_4$ formation rate [6]. However, the formate fluxes are about 100 times higher than the $H_2$ fluxes in both cocultures. These calculations suggest that formate transfer is more important in syntrophic butyrate and propionate degradation than $H_2$ transfer . This conclusion is in accordance with the observations in defined methanogenic cocultures of the two acetogens, where $H_2$/formate-utilizing but not $H_2$-utilizing methanogens can drive butyrate or propionate oxidation [6, 29].

The experiments of the bicarbonate effect on formate and $H_2$ formation from butyrate and propionate suggested that $H_2$ and formate were produced simultaneously during butyrate and propionate oxidation, i.e. reducing equivalents are used either to reduce protons or to reduce $HCO_3^-$. Although *S. bryantii* and MPOB are able to interconvert $H_2$ + $HCO_3^-$ and formate, this process seems to be relatively unimportant during syntrophic propionate and butyrate oxidation. The concentrations at which this interconversion occurs are far higher than the $H_2$ and formate concentrations which are required for syntrophic degradation. If this interconversion would occur at low concentrations, $H_2$-utilizing methanogens would have been able to drive propionate and butyrate oxidation. Clearly, this was not the case [6, 29].

## Acknowledgement

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Table 5. Calculated $H_2$ and formate flux in cocultures of *M. hungatei* with MPOB and with *S. bryantii* respectively using the Fick's diffusion equation[a]

| | *S. bryantii* | MPOB |
|---|---|---|
| Cell size of acetogen(μm) | 0.4*4.5-6 [10] | 1.1-1.6*1.8-2.5 [16] |
| Cell numbers in coculture at end of growth | $1.3*10^9$ | $9.6*10^8$ |
| Cell surface area of acetogen(μm²) | $1.7*10^9$ | $1.8*10^9$ |
| Average distance between cells(μm) | 9.16 | 12.4 |
| Measured $CH_4$ formation of coculture ($nmol.min^{-1}. ml^{-1}$) | 0.77 [6] | 0.42 [29] |
| Highest $H_2$ formed by acetogen(Pa) | 171 | 6.8 |
| Highest formate formed by acetogen(μM) | 280 | 24 |
| Lowest $H_2$ reached by *M. hungatei*(Pa) | 2 [27] | 2 |
| Lowest formate reached by *M. hungatei*(μM) | 15 [28] | 15 |
| $H_2$ flux($nmol.min^{-1}. ml^{-1}$) | 50.4 | 1.13 |
| Formate flux($nmol.min^{-1}. ml^{-1}$) | 4422 | 117 |

a: the numbers in square brackets indicate the literature references.

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# Chapter 5

# Localization of the enzymes involved in $H_2$ and formate metabolism in *Syntrophospora bryantii*

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## Abstract

Cell free extracts of crotonate-grown cells of the syntrophic butyrate-oxidizing bacterium-*Syntrophospora bryantii* contained high hydrogenase activities (8.5-75.8 $\mu$mol. min$^{-1}$. mg$^{-1}$ protein) and relatively low formate dehydrogenase activities (0.04-0.07 $\mu$mol. min$^{-1}$. mg$^{-1}$ protein). The $K_M$ value and threshold value of the hydrogenase for $H_2$ were 0.21 mM and 18 $\mu$M, respectively, whereas the $K_M$ value and threshold value of the formate dehydrogenase for formate were 0.22 mM and 10 $\mu$M, respectively. Hydrogenase, butyryl-CoA dehydrogenase and 3-OH-butyryl-CoA dehydrogenase were detected in cytoplasmic fraction. Formate dehydrogenase and $CO_2$ reductase were membrane bound, likely located at the outer aspect of the cytoplasmic membrane. Results suggest that during syntrophic butyrate oxidation, $H_2$ is formed intracellularly, while formate is formed at the outside of the cell.

*Key words*: anaerobic degradation, butyryl-CoA dehydrogenase, 3-OH-butyryl-CoA dehydrogenase, electron transfer, formate dehydrogenase, hydrogenase, *Syntrophospora bryantii*

## Introduction

*Syntrophospora bryantii* (Stieb & Schink 1985; Zhao et al. 1990) is a gram-positive spore-forming anaerobic bacterium which oxidizes butyrate to acetate and $H_2$ and/or formate. Because of unfavorable energetics, *S. bryantii* can degrade butyrate only if $H_2$ and/or formate is efficiently removed by methanogens. However, *S. bryantii* is able to grow in pure culture by fermentation of crotonate to butyrate and acetate (Zhao et al. 1990) and by oxidation of butyrate to acetate with pentenoate as the terminal electron acceptor (Dong et al. 1994).

Research with *S. bryantii* and another butyrate oxidizer, *Syntrophomonas wolfei*, has shown that butyrate is oxidized by ß-oxidation via the crotonyl-CoA pathway (Schink 1992; Wofford et al. 1986). In this pathway, butyrate is activated to butyryl-CoA by a butyrate: acetyl-CoA HSCoA transferase, which then is oxidized to two acetyl-CoA. One acetyl-CoA is used to activate butyrate, while the other is converted to acetate via acetyl-phosphate, resulting in the formation of 1 ATP. The reducing equivalents are released at the energetic level of $FADH_2$ and NADH during the oxidation of butyryl-CoA to crotonyl-CoA and of 3-OH-butyryl-CoA to acetoacetyl-CoA, respectively. The oxidation of NADH and particularly of $FADH_2$ coupled to $H_2$ and formate formation (i.e. proton and $CO_2$ reduction) is energetically unfavorable. NADH oxidation becomes possible at low $H_2$ and formate concentrations. However, for $FADH_2$ oxidation the organism has to invest ATP to drive this reaction even at low $H_2$ and formate concentrations. A reversed electron flow mechanism has been proposed for this (Thauer & Morris 1984; Schink 1992).

At present it is still not clear whether $H_2$ transfer or formate transfer is most important in the syntrophic degradation of butyrate by *S. bryantii*. In a previous study we have found that *S. bryantii* can oxidize butyrate in coculture with methanogens that can use $H_2$ and formate, but not in coculture with methanogens that can only use $H_2$ (Dong et al. 1994). In addition, *S. bryantii* was able to produce both $H_2$ and formate during butyrate oxidation (unpublished results). The aim of this study was to elucidate how intracellularly reducing equivalents released during butyrate oxidation are channelled to the terminal electron acceptors $H^+$ and $HCO_3^-$.

## Materials and methods

### *Organism and cultivation*

The biculture of *Syntrophospora bryantii* and *Methanospirillum hungatei* (DSM 3014B) was obtained from the German culture collection of microorganisms (DSM, Braunschweig, Germany). *S. bryantii* was isolated by us from the biculture by using media with sodium crotonate (2.2 g/l) as carbon source. This was done as described by Zhao et al. (1990). The bacterium was cultivated in 1-liter bottles in a medium as described previously (Dong et al. 1994).

### *Preparation of cell extracts and cell fractionation*

Unless indicated otherwise, all the manipulations were done in an anaerobic glove box with a $N_2/H_2$ (96:4) atmosphere. Traces of oxygen were removed by a palladium catalyst (BASF, Arnhem, The Netherlands). Crotonate-grown cells were harvested in the late-log-phase by anaerobic centrifugation at 10,000 g for 15 min., and the cell pellet was suspended in 100 mM Tris-HCl buffer (pH 7.5) containing 250 mM sucrose, 20 mM KCl, 5 mM $MgCl_2$ and 5 mM dithiothreitol (DTT). The cell suspension was passed twice through a French pressure cell at 40 MPa. Unbroken cells and cell debris were removed by centrifugation at 11,000 g at 4°C for 20 min.. The supernatant was designated as cell-free extract. For fractionation into a cytoplasmic fraction and a membrane fraction, the cell-free extract was ultracentrifuged at 140,000 g at 4°C for 1.5 h. The pellet designated as membrane fraction was resuspended in the same buffer as described above.

Cell fractionation was also done after an osmotic shock treatment. For this 1.5 g cells (wet weight) were suspended in 10 ml 50 mM Tris-HCl buffer (pH 7.6) containing 0.5 M sucrose. A lysozyme solution was added (50 $\mu$g/ml, final concentration), and the suspension was incubated for 30 min. at room temperature. Thereafter, the suspension was centrifuged at 17,500 g at 4°C for 20 min.. The spheroplast pellet was resuspended in the same buffer as described above but without sucrose to produce a "spheroplast lysate". A membrane fraction and a cytoplasmic fraction were obtained by centrifuging at 17,500 g at 4°C for 20 min.. The preparations were stored under $N_2$ in 9-ml serum bottles at 4°C. All the enzyme activities were analyzed within 8 hours after preparation.

### *Enzyme assays*

All the enzyme assays were performed anaerobically under $N_2$ ($H_2$ for hydrogenase assay) at 37°C in 1-cm cuvettes sealed with rubber stoppers. The reduction of electron acceptors and the oxidation of electron donors were followed spectrophotometrically using a Beckman DU 7500 spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme catalyzing the conversion of 1 $\mu$mol substrate per min.. Protein content was determined using the method of Bradford (1976).

Benzyl viologen-coupled hydrogenase and benzyl viologen-coupled formate dehydrogenase activities were measured at pH 8.0 as described by Lamed & Zeikus (1980) and Leonhart & Andreesen (1977), respectively. Benzyl viologen-coupled $CO_2$ reductase was determined at pH 6.8 according to Thauer et al. (1973). MTT-coupled NADH dehydrogenase was measured in 50 mM Hepes-KOH buffer (pH 7.5) as described by Bergsma et al. (1982). Dichlorophenolindophenol (DCPIP)-coupled butyryl-CoA dehydrogenase and NADH-dependent 3-OH-butyryl-CoA dehydrogenase were assayed as described by Wofford et al. (1986). ATPase was measured by the method of Vogel & Steinhart (1976). NAD-, NADP-, FMN-, FAD- and ferredoxin-dependent hydrogenase and formate dehydrogenase were measured at pH 7.5 as described by Schauer & Ferry (1982). The following extinction coefficients were used: NAD and NADP, $\epsilon_{334}$=6.18 $mM^{-1}.cm^{-1}$; FMN, $\epsilon_{450}$=41 $mM^{-1}.cm^{-1}$; FAD, $\epsilon_{450}$=44.3 $mM^{-1}.cm^{-1}$; ferredoxin, $\epsilon_{390}$=16 $mM^{-1}.cm^{-1}$. Benzyl viologen, $\epsilon_{578}$=9.2 $mM^{-1}.cm^{-1}$; DCPIP, $\epsilon_{600}$=21 $mM^{-1}.cm^{-1}$ and MTT, $\epsilon_{578}$=13 $mM^{-1}.cm^{-1}$.

### *Chemicals*

Enzymes and cofactors for enzyme assays were obtained from Boehringer Mannheim (Almere, The Netherlands). The chemicals which were at least of analytical grade were obtained from Sigma Chemical Co. (Amsterdam, The Netherlands) or Merck (Darmstadt, Germany). Butyryl-CoA and δ-acetoacetyl-CoA were purchased from Aldrich Chemie (Brussel, Belgium). Phenazine methosulfate (PMS) was obtained from Janssen chimica (Tilburg, The Netherlands) and dichlorophenolindophenol (DCPIP) from Fluka Chemika-Biochemika (Breda, The Netherlands).

## Results

### *Hydrogenase and formate dehydrogenase activities in cell extract of S. bryantii*

Extracts of *S. bryantii* cells grown on crotonate contained very high hydrogenase activities (Table 1). In the several experiments which had been done the hydrogenase activity was never below 8.5 $\mu$mol.min$^{-1}$.mg$^{-1}$. In contrast formate dehydrogenase activities were lower (Table 1). The highest formate dehydrogenase activity measured in crude extracts was 0.07 $\mu$mol.min$^{-1}$.mg$^{-1}$. The $V_{max}$ values, the $K_M$ values and the threshold values of the two enzymes are shown in table 1. The activities of hydrogenase and formate dehydrogenase of cells grown on pentenoate plus butyrate and of cells grown on crotonate plus formate were in the same range as the activities in crotonate-grown cells ( data not shown).

Table 1. Hydrogenase and formate dehydrogenase activites measured in cell extracts of *S. bryantii* [a]

| activity | $K_M$ (mM) | $V_{max.}$ ($\mu$mol.min$^{-1}$.mg$^{-1}$) | Threshold value[b] ($\mu$M) |
|---|---|---|---|
| hydrogenase | 0.21 | 8.5-75.8 | 18 |
| formate dehydrogenase | 0.22 | 0.04-0.07 | 10 |

a: All values refer to $H_2$ and formate concentrations in the liquid. It was assumed that the soluble $H_2$ concentration was 0.59 mM when $10^5$ Pa $H_2$ was present in the gas phase (Boone et al. 1989).

b: These values refer to the lowest concentration at which activity could be measured in the standard assay.

### *Enzyme activities of intact cells and of cell fractions prepared by French pressure lysis*

The enzymatic activities in the different cell fractions prepared by French press disrupture are listed in Table 2. Some activities could be measured with whole cells. Benzyl viologen-coupled hydrogenase activity and MTT-coupled NADH dehydrogenase activity increased upon cell lysis. However, the benzyl viologen-coupled formate dehydrogenase activity of whole cells was the same as the activity in cell extracts. Upon fractionation of the crude extracts, up to 95% of the hydrogenase activity and 75% the NADH dehydrogenase activity were found in the soluble fraction, while up to 50% of the formate dehydrogenase activity was recovered in the membrane fraction. About 90% of the DCPIP-coupled butyryl-CoA dehydrogenase and the NAD-coupled 3-OH-butyryl-CoA dehydrogenase activities were determined in the soluble fraction of the cell extract. Benzyl viologen-coupled $CO_2$ reductase activity was only found in the membrane fraction. Upon fractionation 88% of the presumably membrane-bound ATPase was recovered in soluble fraction. This indicates that cell breakage through French press releases some membrane-bound proteins into the soluble fraction.

### *Enzyme activities in membrane and cytoplasm fraction prepared by osmotic shock lysis*

Table 3. shows that the ATPase activity was totally recovered in the membrane fraction when the osmotic shock method was applied. This indicates that cell breakage by this method is much milder than by the French pressure method. Butyryl-CoA dehydrogenase and 3-OH-butyryl-CoA dehydrogenase activities again were mainly detected in the cytoplasmic fraction. Hydrogenase and formate dehydrogenase activities were recovered in the membrane fraction

Table 2. Comparison of enzymes activity of *S. bryantii* grown on crotonate in different cell fractions prepared after French press lysis[a]

| activity | | Whole cells | Crude extracts | | Membranes | | Soluble fraction | |
|---|---|---|---|---|---|---|---|---|
| | | sp. act. | sp. act. | total U | sp. act. | %U | sp. act. | %U |
| hydrogenase | (1) | 3.5 | 19.8 | 4036 | 4.6 | 7.8 | 38.9 | 95 |
| | (2) | 2.8 | 8.5 | 785 | 22.7 | 9.8 | 54.9 | 88 |
| | (3) | 39.9 | 75.8 | 3304 | 11.7 | 4 | 152 | 92.3 |
| formate dehydrogenase | (1) | 0.06 | 0.04 | 4.9 | 0.1 | 36 | 0.04 | 32.4 |
| | (2) | 0.07 | 0.07 | 6.4 | 0.39 | 50.7 | 0.05 | 19.1 |
| | (3) | 0.04 | 0.04 | 7.8 | 0.16 | 34.3 | 0.02 | 21.4 |
| $CO_2$ reductase | (1) | 0.02 | ND | | 0.014 | 100 | 0 | 0 |
| NADH dehydrogenase | (1) | 0.88 | 4.0 | 814 | 1.04 | 0.88 | 5.85 | 75.5 |
| butyryl-CoA dehydrogenase | (1) | ND | ND | | 0.08 | 10 | 0.3 | 90 |
| 3-OH-butyryl-CoA dehydrogenase | (1) | ND | ND | | 4.5 | 8.8 | 17.8 | 91 |
| ATP-ase | (1) | ND | ND | | 0.15 | 12 | 0.08 | 88 |

a: The numbers in parentheses refer to experiments with different batches of cells; ND means not determined.

Table 3. Comparison of enzyme activities in soluble and membrane fractions prepared after osmotic shock lysis

| Activity | Crude extract | | Membrane fraction | | soluble fraction | |
|---|---|---|---|---|---|---|
| | Spec. activity | total U | Spec. activity | % total U | Spec. activity | % total U |
| Hydrogenase | 63 | 5708 | 94.2 | 82.3 | 25.0 | 17.7 |
| Formate dehydrogenase | 0.33 | 29.7 | 0.43 | 72.4 | 0.2 | 27.6 |
| Butyryl-CoA dehydrogenase | 0.75 | 70 | 0.22 | 16 | 1.46 | 84 |
| 3-OH-butyryl-CoA dehydrogenase | 21.9 | 1977 | 14.6 | 36.8 | 30.9 | 63.2 |
| ATP-ase | 23.7 | 1182 | 23.7 | 100 | 0 | 0 |

Table 4. Specific activities ($\mu$mol.min$^{-1}$.mg$^{-1}$) of hydrogenase and formate dehydrogenase of *S. bryantii* coupled to different electron acceptors

| electron acceptors | hydrogenase (cytoplasm) | formate dehydrogenase (membrane) | $E^{\circ\prime}$ (mV) |
|---|---|---|---|
| Ferredoxin | 18.2 | 0.24 | -398 |
| Benzyl viologen | 152 | 0.43 | -360 |
| NAD | 11.7 | 0.07 | -320 |
| NADP | 0.06 | <0.01 | -324 |
| FAD | 1.3 | <0.01 | -220 |
| FMN | 0.7 | <0.01 | -190 |
| $NADH+H^{+} -> H_2 + NAD^{+}$ | 0.012 | | |

for 82% and 72%, respectively. The specific activities of hydrogenase and formate dehydrogenase in the cell lysate after osmotic shock treatment were 1.5-8 times higher than after French pressure lysis. Butyryl-CoA dehydrogenase and 3-OH-butyryl-CoA dehydrogenase activities were also 3-5 times higher, both in the membrane fraction and in the soluble fraction, than those prepared by French pressure. ATPase activity in the membrane fraction was up to 160 times higher when cells were disrupted by osmotic shock.

### *Hydrogenase and formate dehydrogenase of S. bryantii coupled to different electron acceptors*

Different electron carriers could be used in the hydrogenase and the formate dehydrogenase assays (Table 4). Hydrogenase could be assayed with NAD, FAD or FMN and ferredoxin. However, the activities were much lower than with benzyl viologen. Formate dehydrogenase could also be assayed with NAD and ferredoxin, also these activities were lower than when benzyl viologen was used.

## Discussion

Butyrate is degraded by acetogenic bacteria by ß-oxidation via the crotonyl-CoA pathway. The key enzymes of this pathway have been demonstrated in *Syntrophomonas wolfei* (Wofford et al. 1986) and in *S. bryantii* (Schink 1992). The enzyme activities found in this study were in the same range as those reported by Dörner (1992) and by McInerney & Wofford (1992). In butyrate-oxidizing acetogenic bacteria, reducing equivalents released in the oxidation of butyryl-CoA to crotonyl-CoA and the oxidation of 3-OH-butyryl-CoA to acetoacetyl-CoA have to be channelled to protons and/or to bicarbonate, resulting in the formation of $H_2$ and formate. To get insight into the intracellular electron flow we determined the localization of redox proteins in *S. bryantii*. For this purpose we used two different cell lysis methods, the French pressure method and the osmotic shock lysis method. It should be mentioned that results of enzyme localization measurements have to be interpreted with caution because membrane-associated enzymes can be recovered as soluble proteins, as was the case with the membrane-bound ATPase using French pressure lysis method. An additional complicating factor is that cells of *S. bryantii* lyse rather rapidly after growth has ceased, causing that some intracellular enzymes apparently can be measured with whole cells.

The butyryl-CoA dehydrogenase and 3-OH-butyryl-CoA dehydrogenase activities were mainly found in the cytoplasmic fraction, and the specific activities of the two enzymes in soluble fractions were higher than in the membrane fractions. These results suggest that the two enzymes are located in the cytoplasm. However, the energetically highly unfavorable oxidation of butyryl-CoA coupled to $H_2$ or formate formation in one way or the other has to be associated with the membrane. The butyryl-CoA dehydrogenase was found to be only partly membrane bound in *S. wolfei* (Dörner 1992; Wallrabenstein & Schink 1994). If the butyryl-CoA dehydrogenase is a cytoplasmic protein, a membrane bound $FADH_2$ dehydrogenase has to be present.

The benzyl viologen-coupled hydrogenase activity can be measured with whole cells. However, the activity was increased 2-5 times upon cell breakage by French press lysis. Because benzyl viologen is almost membrane-impermeable, this indicates that the enzyme is mainly intracellularly located. Moreover, the majority of the hydrogenase activity was recovered in the soluble fraction of a French pressure lysate, which also suggests that the hydrogenase of *S. bryantii* is likely located in the cytoplasm. The majority of the hydrogenase was found in the membrane fraction of an osmotic shock lysate, which might indicate that the hydrogenase is partly membrane bound as was also observed in *S. wolfei* (Dörner 1992; Wallrabenstein & Schink 1994). Taking all these observations into account, $H_2$ most likely is formed intracellularly in the cytoplasm or at the inner aspect of the cytoplasmic membrane.

Formate dehydrogenase of *S. bryantii* most likely is a membrane-bound protein which is localized at the outer aspect of the membrane. This conclusion is based on the observation that: i) the formate dehydrogenase activity in the membrane fraction was about 8 times higher than in the cytoplasm fraction, ii) the benzyl viologen-coupled formate dehydrogenase activity was not increased by cell breakage, and iii) the majority of the formate dehydrogenase and all the $CO_2$ reductase were recovered in the membrane fractions prepared either by French pressure lysis or osmotic shock lysis. Additional evidence was obtained in experiments with the ionophores. *S. bryantii* is able to convert formate into $H_2 + CO_2$. This conversion by cell suspensions was not affected by the addition of valinomycin and nigericine, or the ATP-ase inhibitor, N,N-dicyclohexyl-carbodiimide (DCCD) (unpublished results). This indicates either that formate uptake is not energy-dependent or that formate is not taken up at all.

Based on our findings a model is proposed how during syntrophic butyrate oxidation in *S. bryantii* electrons from butyryl-CoA and 3-OH-butyryl-CoA can be channelled to protons to form $H_2$ inside the cell (Fig. 1a) or to bicarbonate to form formate outside the cell (Fig. 1b). The redox potential of crotonyl-CoA/butyryl-CoA couple is -126 mV (Gustafson et al. 1986). Therefore, the oxidation of butyryl-CoA requires the investement of energy whenever the reaction is coupled to proton reduction ($\Delta E^{\circ\prime}{}_{H^+/H_2}$ = -414 mV) or to $HCO_3^-$

reduction ($\Delta E^{\circ\prime}{}_{HCO_3^-/formate} = -432$ mV). It was observed that *S. wolfei* conserved more energy from the fermentation of crotonate than other anaerobes (Beaty & McInerney 1987). The extra amount of energy could come from the reduction of crotonyl-CoA to butyryl-CoA (McInerney & Wofford 1992). A electron transfer mechanism was proposed for butyryl-CoA oxidation in *S. wolfei* (Thauer & Morris 1984; Wallrabenstein & Schink 1994). Part of ATP synthesized via substrate level phosphorylation is used to generate a proton motive force which can be used to drive butyryl-CoA oxidation.

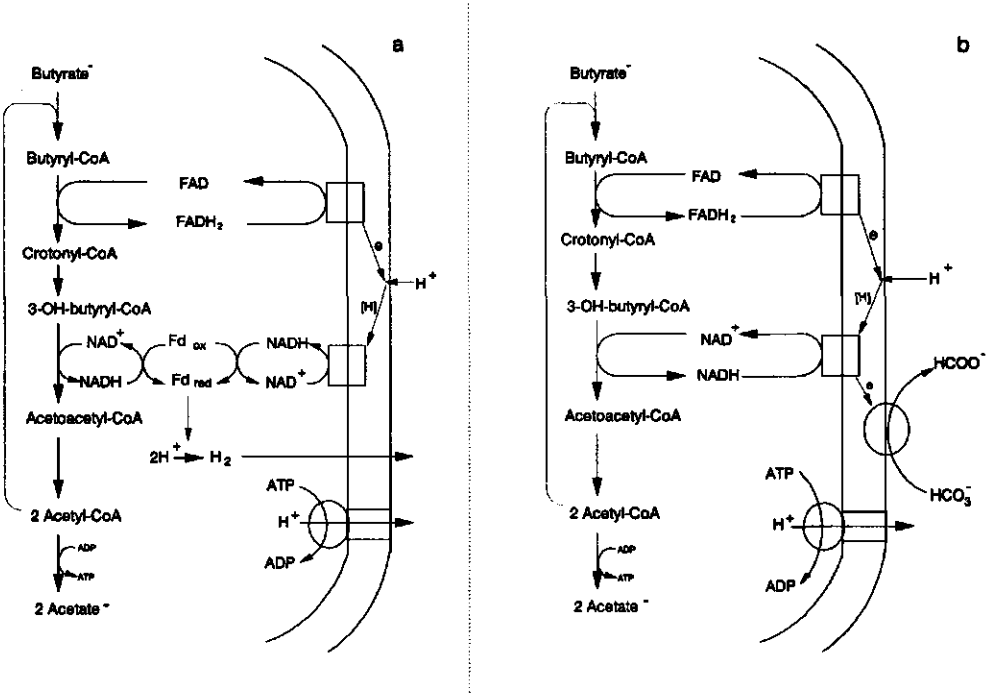

Fig. 1. Hypothetical model of hydrogen formation (a) and formate formation (b) during butyrate oxidation by *Syntrophospora bryantii*.

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# Chapter 6

# Localization of the enzymes involved in the $H_2$ and formate metabolism of a mesophilic propionate-oxidizing bacterium

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## 1. Summary

The mesophilic propionate-oxidizing bacterium (MPOB) contained both hydrogenase and formate dehydrogenase activities, enabling the bacterium to form $H_2$ and formate during syntrophic growth on propionate, and to consume $H_2$ and formate for the reduction of fumarate. The $V_{max}$ and $K_M$ value of hydrogenase for $H_2$ in cell extracts were 1.5-3.7 $\mu mol.min^{-1}.mg^{-1}$ protein and 0.05 mM, respectively, and the $V_{max}$ and $K_M$ value of formate dehydrogenase for formate were 0.2-0.4 $\mu mol.min^{-1}.mg^{-1}$ protein and 0.3 mM, respectively. The localization of the redox enzymes involved in propionate oxidation was determined after cell breakage by French press and by lysozyme treatment. Fumarate reductase was membrane bound and situated at the inner aspect of the membrane, while both malate dehydrogenase and pyruvate dehydrogenase were located in the cytoplasm. Hydrogenase was detected in the cytoplasm, periplasm as well as the membrane fraction; while formate dehydrogenase was detected in the periplasm and the membrane fraction. A model is proposed to explain how intracellularly electrons are channelled to the terminal electron acceptors $H^+$ and $HCO_3^-$.

## 2. Introduction

Bacteria which grow on propionate in syntrophic association with methanogens catabolize propionate to acetate and $CO_2$ via the methylmalonyl-CoA pathway (Koch et al. 1983; Houwen et al. 1990; Plugge et al. 1993). In this pathway propionate is first activated to propionyl-CoA by a propionate kinase and phosphotranspropionylase as in *Syntrophobacter wolinii* (Houwen et al. 1990) or by a propionate:acetyl-CoA HS-CoA transferase as in MPOB (Plugge et al. 1993). Propionyl-CoA is then carboxylated to methylmalonyl-CoA; this reaction is coupled to the decarboxylation of oxaloacetate by means of a transcarboxylase. The methylmalonyl-CoA is further degraded to acetate through several dehydrogenations and decarboxylations. 1 mole of ATP is synthesized via substrate level phosphorylation by conversion of acetyl-CoA to acetate (*S. wolinii)* or by conversion of succinyl-CoA to succinate (MPOB). Reducing equivalents are released from the oxidation of succinate to fumarate, malate to oxaloacetate and pyruvate to acetate, respectively. These reducing equivalents are ultimately transferred to $H^+$ or $HCO_3^-$ to form $H_2$ or formate.

The oxidations of succinate to fumarate and of malate to oxaloacetate are energetically unfavorable reactions ($\Delta G°'$ is 86.2 and 47.7 kJ/mol) when coupled to $H^+$ or $HCO_3^-$ reduction. Therefore, $H_2$ or formate have to be removed to "pull" these oxidation reactions. Up to now it is not clear whether $H_2$ or formate is more important during propionate oxidation, and how reducing equivalents are intracellularly channelled to $H^+$ or $HCO_3^-$ reduction. To elucidate how reducing equivalents in MPOB are coupled to the reduction of $H^+$ or $HCO_3^-$, we determined the localizations of the hydrogenase, formate dehydrogenase and other redox enzymes involved in propionate oxidation.

## 3. Materials and methods

### *3.1. Organisms and cultivation*

The mesophilic propionate-oxidizing bacterium (MPOB) was isolated from a methanogenic propionate-oxidizing culture. It was cultivated in a mineral medium containing sodium fumarate (6.5 g/l) as described previously (Stams et al. 1993).

### *3.2. Preparation of cell fractions*

Cells (2 liters) in the late-log-phase were harvested by anaerobic centrifugation at 10,000 g for 15 min. and suspended in 100 mM Tris-HCl buffer (pH 7.5) with 0.25 M sucrose, 20 mM KCl, 5 mM $MgCl_2$ and 5 mM dithiothreitol (DTT). This suspension was passed twice through a French pressure cell at 40 MPa and the obtained crude extract was centrifuged at 11,000 g at 4°C for 20 min.. The supernatant designated as cell-free extract was ultracentrifuged at 140,000 g for 1.5 h. to get the cytoplasm fraction in the supernatant and the membrane fraction in the pellet. The membrane fraction was resuspended in the same buffer as described above.

Fractionation of cell extract after an osmotic shock treatment was done as described by Odom and Peck (1981). For this, 1.5 g cells (wet weight) were suspended (1:10 wt/vol) in the buffer as described above, but containing 0.5 M sucrose instead of 0.25 M sucrose. This was done in stoppered serum bottles under a $N_2$ gas phase. A lysozyme solution was added (200 $\mu$g/ml, final concentration). The mixture was shaked for 0.5-1 h. at 37°C and then centrifuged at 11,000 g at 4°C for 15 min.. The supernatant was designated as the periplasmic fraction. To get a spheroplast lysate, the spheroplast pellet was resuspened in the same buffer but without sucrose. After 10 min. at room temperature the suspension was centrifuged at 18,000 g at 4°C for 15 min.. The pellet was designated as the membrane fraction and the supernatant as the cytoplasm fraction.

### *3.3 Enzyme assays*

Unless indicated otherwise, all of the enzymes were determined anaerobically at 37°C. The reduction of electron acceptors and the oxidation of electron donors were followed spectrophotometrically using a Beckman DU 7500 spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme catalyzing the conversion of 1 $\mu$mol substrate per min.. Protein content was determined using the method of Bradford (1976).

Benzyl viologen-coupled hydrogenase and formate dehydrogenase were measured at pH 8.0 as described by Lamed and Zeikus (1980), and Leonhart and Andreesen (1977), respectively. Benzyl viologen-coupled $CO_2$ reductase was determined at pH 6.8 according to the method described by Thauer et al. (1973). Benzyl viologen-coupled fumarate reductase was measured as described by Boonstra et al. (1975), malate dehydrogenase was determined according to the method described by Stams et al. (1984), pyruvate dehydrogenase was measured as described by Odom and Peck (1981), and ATPase was measured as described by Vogel and Steinhart (1976).

### *3.4 Cytochrome spectra*

The difference spectra of air-oxidized and dithionite-reduced cytoplasmic and periplasmic fractions were scanned in a 1-cm light path Quartz cuvette on Beckman DU7500 spectrophotometer.

## 4. Results

### *4.1 Hydrogenase, formate dehydrogenase and $CO_2$ reductase activities in cell-free extracts of MPOB:*

The activities of hydrogenase, formate dehydrogenase and $CO_2$ reductase were determined in cell extracts of MPOB. The $V_{max}$, $K_M$ values and the lowest concentration reached by each of the enzymes are listed in table 1. The hydrogenase activity was about 10 times higher than the formate dehydrogenase activity. The $CO_2$ reductase activity was in the same range as the formate dehydrogenase activity. The $K_M$ value of the $CO_2$ reductase for $HCO_3^-$ was 0.43 mM at pH 6.8.

Table 1. Hydrogenase, formate dehydrogenase and $CO_2$ reductase activities in cell extract of MPOB[a]

| activity | $K_M$(mM) | $V_{max}$($\mu$mol.min$^{-1}$.mg$^{-1}$.) | lowest concentration($\mu$mol) |
|---|---|---|---|
| Hydrogenase | 0.05 | 1.5-3.7 | 6 |
| Formate dehydrogenase | 0.3 | 0.2-0.4 | 70 |
| $CO_2$ reductase | 10.5 | 0.15-0.23 | 430 |

a: The values refer to $H_2$ concentration in liquid for hydrogenase, formate concentration for formate dehydrogenase and bicarbonate concentration for $CO_2$ reductase, respectively.

### *4.2 Enzyme activities of intact cells and of cell fractions prepared by French pressure disrupture:*

Fumarate reductase (succinate dehydrogenase), malate dehydrogenase and pyruvate dehydrogenase catalyze the dehydrogenation reactions involved in propionate oxidation. Table 2 shows that their activities were significantly increased by cell lysis, indicating their intracellular localization. Upon fractionation of the cell extract, the majority of the fumarate reductase activity was measured in membrane fraction. While both the malate dehydrogenase and the pyruvate dehydrogenase were determined as cytoplasmic enzymes.

Benzyl viologen-coupled hydrogenase activities were increased 1.2 to 2 times upon cell breakage, while the benzyl viologen-coupled formate dehydrogenase activity in cell extracts was the same as that of whole cells. After fractionation of the cell extracts the majority of the hydrogenase and the formate dehydrogenase was recovered in the soluble fraction, while only up to 18% and 31% of the activities were detected in the membrane fraction. However, 30% of the ATPase activity, a presumably membrane bound protein, was determined in the soluble fraction as well. This indicates that membrane bound proteins could be partly released from the membrane with the French pressure method. Treatment of the membrane fraction with 0.1% triton increased the formate dehydrogenase activity by 30% (data not shown).

### *4.3 Enzyme activities in different cell fractions prepared after osmotic shock lysis:*

Osmotic shock lysis is a milder method than the French pressure method as indicated by the fact that both the activity and the distribution of the ATPase are higher in the

Table 2. Comparison of enzymes activity of MPOB grown on fumarate in different cell fractions prepared by French pressure lysis[a]

| activity | | Whole cell | crude extract | | membrane fraction | | soluble fraction | |
|---|---|---|---|---|---|---|---|---|
| | | sp. act. | sp.act. | total U | sp.act. | % total U | sp.act. | % total U |
| Hydrogenase | (1) | 1.0 | 1.5 | 91 | 0.6 | 18 | 0.8 | 74 |
| | (2) | 2.1 | 4.0 | 143 | 3.4 | 18 | 4.4 | 88 |
| | (3) | 2.1 | 2.5 | 54 | 0.7 | 2.5 | 1.8 | 41 |
| Formate dehydrogenase | (1) | 0.5 | 0.4 | 21.8 | 0.1 | 13 | 0.2 | 67 |
| | (2) | 0.2 | 0.2 | 15.8 | 0.1 | 16 | 0.5 | 84 |
| | (3) | 0.2 | 0.2 | 75 | 0.4 | 31 | 0.2 | 70 |
| Fumarate reductase | (1) | 0.04 | 1.4 | 121 | 3.5 | 77 | 0.4 | 8 |
| | (2) | 0.05 | 1.6 | 55 | 6.3 | 88 | 0.3 | 12 |
| Malate dehydrogenase | | 0.6 | ND | | 0 | 0 | 40.8 | 100 |
| Pyruvate dehydrogenase | | 0.02 | ND | | 0.08 | 11.6 | 0.6 | 88.4 |
| ATPase | | ND | 1.5 | 54.6 | 3.9 | 55 | 0.6 | 30 |

a: The numbers in parentheses refer to experiments with different batches of cells; ND means not determined.

Table 3. Distribution of viologen-coupled enzyme activities in cell fractions prepared after lysozyme lysis[a]

| activity | | Periplasm | | membrane | | cytoplasm | |
|---|---|---|---|---|---|---|---|
| | | sp.act. | %total U | sp.act. | %total U | sp.act. | %total U |
| Hydrogenase | (1) | 5.0 | 38 | 8.2 | 44 | 3.8 | 18 |
| | (2) | 4.2 | 34 | 1.9 | 31 | 1.0 | 22 |
| Formate dehydrogenase | (1) | 0.4 | 36 | 1.2 | 53 | 0.3 | 11 |
| | (2) | 0.8 | 41 | 0.2 | 31 | 0.2 | 28 |
| Fumarate reductase | (1) | 0.8 | 16 | 4.5 | 67 | 1.3 | 17 |
| | (2) | 2 | 16 | 4.2 | 90 | 0.3 | 7 |
| Malate dehydrogenase | | 1.6 | 20 | 0.9 | 48 | 1.4 | 32 |
| Pyruvate dehydrogenase | | 13.5 | 26 | 6.8 | 36 | 7.8 | 39 |
| ATPase | | 15.2 | 10 | 45.2 | 84 | 3.8 | 7 |

a: The numbers in parentheses refer to experiments with different batches of cells membrane fraction after the osmotic shock treatment than after the French pressure treatment. Table 3 shows that up to 38 and 36 percent of hydrogenase and formate dehydrogenase activities were determined in the periplasm fraction, respectively; while the percentage of the two enzymes in the membrane fraction was higher after the osmotic shock treatment. Only 16 and 10 percent of the fumarate reductase and ATPase, which are presumbly membrane-bound proteins, were measured in the periplasmic fraction. However, a certain amount of the malate dehydrogenase and the pyruvate dehydrogenase, which are thought to be cytoplasmic proteins, was also found in the periplasmic fraction.

### *4.4 Cytochromes in cytoplasm and periplasm*:

Reduced (dithionite) versus oxidized spectra of cytoplasmic and periplasmic fractions of MPOB showed spectra which are typical for cytochrome $C_3$; maxima were found at 420, 522 and 553 nm.

## 5. Discussion

Enzyme assays showed that MPOB contained all the enzyme activities of the methylmalonyl-CoA pathway involved in propionate oxidation (Plugge et al. 1993). In this pathway, three oxidation reactions release 3 couples of electrons which ultimately have to be coupled to the reduction of $H^+$ or $HCO_3^-$ leading to the formation of $H_2$ or formate, respectively. To know how the electrons are channelled to the terminal electron acceptors, we have determined the localization of the redox enzymes involved in propionate oxidation.

Fumarate reductase most likely is a membrane bound protein located at the inner aspect of the membrane. Malate dehydrogenase and pyruvate dehydrogenase are detected as cytoplasmic enzymes, upon fractionation of the cell extract prepared by French pressure disrupture. However, a relative high percentage of their activities was also detected in the membrane fractions prepared by osmotic shock. It is unclear whether they are indeed partly membrane-bound.

From the results it is difficult to conceive where the hydrogenase and the formate dehydrogenase are located. MPOB is a gram negative anaerobe, belonging to the sulfate-reducing bacteria based on 16S rRNA sequence analysis (Harmsen, personnal communication ). As in *Desulfovibrio gigas* (George et al. 1974), up to 38 percent of the hydrogenase activity of MPOB was detected in the periplasmic fraction upon fractionation of the cell extract prepared by osmotic shock treatment, indicating that the hydrogenase of MPOB is partly located in periplasm. However, the benzyl viologen coupled-hydrogenase activity increased up to 2 times upon cell disrupture using the French pressure method, suggesting its intracellular location because benzyl viologen is thought to be membrane-impermeable. Upon fractionation of the cell extract prepared either by French pressure or by osmotic shock, the majority of the hydrogenase was recovered in the soluble fraction and only a low percentage was found in the membrane fraction, indicating that the hydrogenase might be largely located in the soluble fractions, both in the periplasm and the cytoplasm. The benzyl viologen-coupled formate dehydrogenase activity was not affected by cell lysis. Although up to 84 percent of the activity is recovered in the soluble fraction upon fractionation of the cell extract prepared by the French pressure method, a high percentage of the formate dehydrogenase was recovered in the membrane fraction and the periplasm fraction when the osmotic shock treatment was applied. Moreover, treatment of the membranes with a detergent increased the formate dehydrogenase activity to some extent. These observations suggest that the formate dehydrogenase is partly located in the periplasm and partly membrane bound, most likely situated on the outer aspect of the membrane. Some evidence for the external localization of the formate dehydrogenase can be obtained from formate uptake experiments. MPOB is able to convert formate into $H_2 + CO_2$. This conversion was not inhibited by the addition of the ionophores, valinomycin or nigericine, and the ATPase inhibitor, N,N-dicyclohexyl carbodiimide (DCCD) (unpublished results). These observations indicate that formate uptake is either an energy-independent process in MPOB, or that formate is not taken up at all.

Based on the localization of the redox enzymes, a hypothetical model how intracellularly electrons are channelled to $H^+$ and $HCO_3^-$ can be given (Fig. 1). Our hypothesis is that formate is formed at the outer aspect of the membrane, while where $H_2$ is formed is unclear yet. It is assumed that the periplasmic hydrogenase of *Desulfovibrio* sp. responsible for $H_2$ oxidation, while the cytoplasmic hydrogenase is responsible for $H_2$ production from lactate or ethanol oxidation (Badziong and Thauer 1980; George et al. 1974; Odom and Peck 1981). In analogy with this it seems likely that MPOB forms $H_2$ from NADH and reduced ferredoxin intracellularly.

The oxidation of succinate to fumarate is highly endergonic ($E^{\circ\prime}_{fumarate/succinate} = +33$ mV) when the reaction is coupled to $H^+$ or $HCO_3^-$ reduction ($E^{\circ\prime}_{H^+/H_2} = -414$mV and $E^{\circ\prime}_{HCO_3^-/HCOO^-} = -432$ mV). Therefore, metabolic energy has to be invested to pull this oxidation.

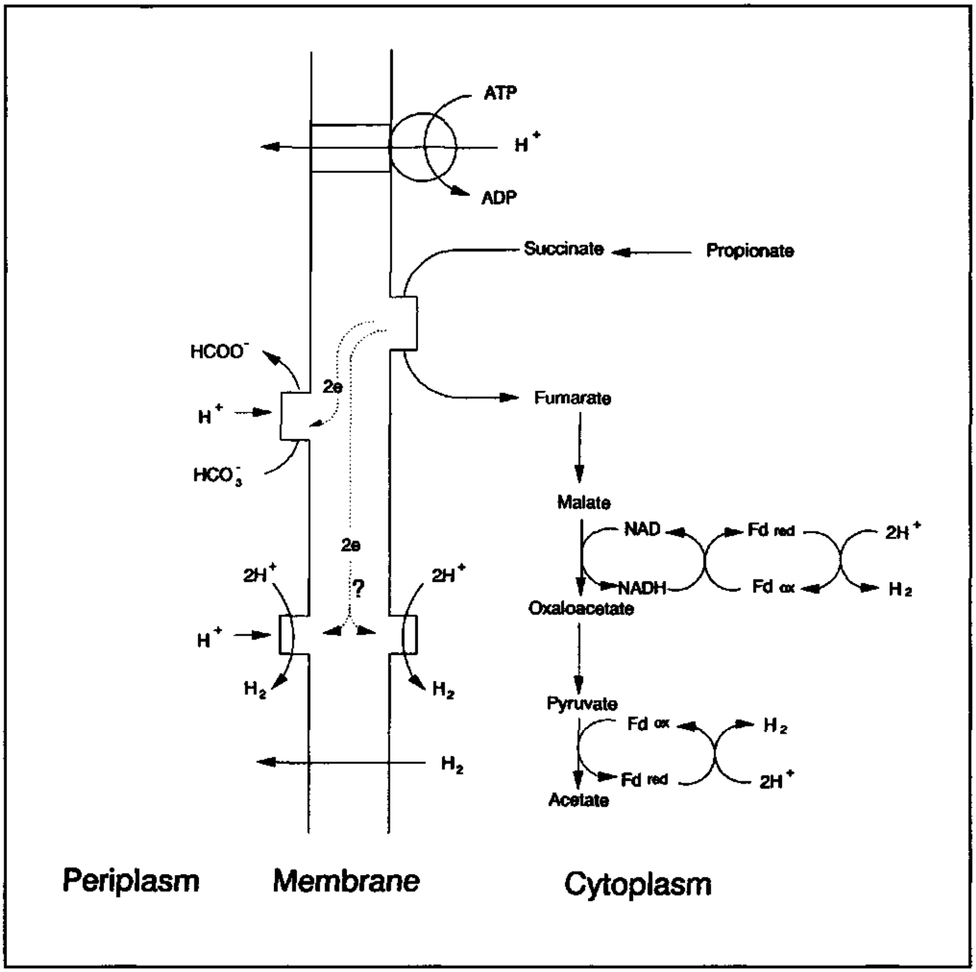

Fig. 1. A hypothetical reversed electron transport mechanism during syntrophic propionate oxidation by MPOB. The oxidation of succinate is completed by a membrane bound fumarate reductase; the electrons are transducted either to formate dehydrogenase, which is situated at the outer aspect of the membrane or to hydrogenase, which might be located at the inner aspect or outer aspect of the membrane. In the scheme, NADH and reduced ferredoxin oxidations are coupled to the intracellular formation of $H_2$. However, it might also be that the oxidation of these electrons is linked to a membrane-bound $CO_2$ reductase in an unknown fashion.

A reversed electron transport mechanism was postulated (Schink 1992; Stams 1994); part of the ATP synthesized at substrate level is spent to generate a proton motive force which can be used to drive succinate oxidation. A study with *Wolinella succinogenes* revealed that the fumarate reduction with $H_2$ and formate as electron donors is a membrane-associated process (Kröger 1978). The externally electrons released from $H_2$ or formate are channelled to the intracellular fumarate reductase, thereby creating a proton motive force. ATP is formed from the $H^+$ gradient across the membrane by means of the ATPase. It has been shown that this bacterium forms 2/3 ATP per fumarate.

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# Chapter 7

# Summary

Under methanogenic conditions, complex organic matter is mineralized by fermentative, acetogenic and methanogenic bacteria. Propionate and butyrate are two important intermediates; they account for 35% and 8% of the total methane formation, respectively. Propionate and butyrate are converted to $CH_4$ and $CO_2$ by the syntrophic consortia of acetogenic and methanogenic bacteria. Both $H_2$ transfer and formate transfer were proposed as the possible mechanisms by which reducing equivalents are transferred from the acetogens to the methanogens. Since extreme low levels of $H_2$ and formate prevail in anaerobic ecosystems and cocultures, it is not known which of the two is more important.

The aim of this research was to clarify the relative importance of $H_2$ and formate transfer during syntrophic degradation of propionate and butyrate by means of physiological and biochemical approaches.

After succeeding in obtaining pure cultures of propionate oxidizer MPOB and butyrate oxidizer *Syntrophospora bryantii* by growing them on fumarate and crotonate, respectively, this study became possible. By constructing defined methanogenic cocultures, it was observed that propionate oxidation by MPOB and butyrate oxidation by *S. bryantii* can only be driven by $H_2$/formate- but not by $H_2$-utilizing or aceticlastic methanogens (Chapter 2 and 3), indicating that formate transfer might be prevailing in these cultures. However, a triculture of MPOB with a *Desulfovibrio* strain, which converts formate into $H_2/CO_2$, and a $H_2$-utilizing methanogen also degraded propionate, confirming that $H_2$ transfer is possible provided that low formate concentrations are maintained (Chapter 2).

MPOB is able to ferment fumarate to succinate and to couple propionate oxidation to fumarate reduction; while *S. bryantii* is able to couple butyrate oxidation to the reduction of pentenoate (Chapter 3). With a limiting amount of fumarate in a propionate degrading culture, the maximum $H_2$ and formate levels produced from propionate by MPOB were 6.8 Pa and 24 $\mu$mol, respectively; and with a limiting amount of pentenoate in a butyrate degrading culture, the maximum $H_2$ and formate levles produced by *S. bryantii* were 170 Pa and 280 $\mu$mol, respectively (Chapter 4). These results showed that both $H_2$ and formate indeed were formed and that the values which were reached were in the range which could be expected from thermodynamical consideration. Using a diffusion model it could be calculated that formate fluxes can be 100 times higher than $H_2$ fluxes in the methanogenic cocultures of MPOB and *S. bryantii*, suggesting that formate transfer is more important during syntrophic propionate and butyrate degradation.

Enzyme measurments (Chapter 5) showed that *S. bryantii* contained very high hydrogenase activity and low formate dehydrogenase activity. The $K_m$ values of the two enzymes are about the same, 0.21 mM and 0.22 mM, respectively. Butyryl-CoA dehydrogenase and 3-hydroxybutyryl-CoA dehydrogenase, which are involved in the two oxidation reactions during butyrate oxidation, were determined as soluble enzymes. The hydrogenase was also determined as a cytoplasmic enzyme while formate dehydrogenase and $CO_2$-reductase were membrane bound, likely located at the outer aspect of the cytoplasmic membrane. These observations suggest that during butyrate oxidation $H_2$ is formed intracellularlly, while formate is formed extracellularly. A reversed electron transfer mechanism was postulated to drive the endergonic oxidation of butyryl-CoA to crotonyl-CoA. Similar experiments were done with MPOB (Chapter 6). MPOB also contained relative high hydrogenase activities (1.5-3.7 $\mu$mol.min$^{-1}$.mg$^{-1}$) and low formate dehydrogenase activities (0.2-0.4 $\mu$mom.min$^{-1}$.mg$^{-}$

$^{1}$). The $K_m$ values are 0.05 mM of $H_2$ for hydrogenase and 0.3 mM of formate for formate dehydrogenase. Localization of the redox enzymes involved in propionate oxidation indicated that the fumarate reductase was membrane bound, likely located at the inner aspect of membrane; while malate dehydrogenase and pyruvate dehydrogenase were located in the cytoplasm. The hydrogenase is located partly in the periplasm, partly in the cytoplasm and partly in the membrane; while the formate dehydrogenase was determined partly in the periplasm and partly in the membrane. These results indicate that, during propionate oxidation, formate is formed outside of cytoplasmic membrane, However, where $H_2$ is produced is not clear yet. As with butyrate oxidation, a reversed electron transport mechanism was postulated to drive the oxidation of succinate ($\Delta G^{\circ\prime} = +86.2$ kJ/mol).

Although acetate is also a product of propionate and butyrate oxidation, removal of acetate by aceticlastic methanogen alone does not drive the oxidations by the acetogens MPOB and *S. bryantii*. However, addition of the aceticlastic methanogen *Methanothrix (Methanosaeta) soehngenii* increased the degradation rates of propionate and butyrate by the cocultures of MPOB and *S. bryantii* with $H_2$/formate-trophic methanogens (Chapter 2 and 3). The addition of 50 mM acetate decreased the butyrate degradation rate of the coculture of *S. bryantii* and *Methanospirillum hungatei* by about 60% (Chapter 3). These results indicate that a low acetate concentration is beneficial for syntrophic degradation of propionate and butyrate.

## Chapter 8

# Samenvatting

Complex organisch materiaal wordt onder anaërobe methanogene omstandigheden afgebroken door consortia van fermentatieve, acetogene en methanogene bacteriën. Propionaat en butyraat zijn twee belangrijke intermediairen; zij dragen respectievelijk voor 35% en 8% bij aan de totale hoeveelheid gevormd methaan. Propionaat en butyraat worden omgezet naar $CH_4$ en $CO_2$ door syntrofe consortia van acetogene en methanogene bacteriën. Zowel de overdracht van $H_2$ als van formiaat worden genoemd als mogelijke mechanismen waarmee reductie-equivalenten worden overgedragen van acetogene bacteriën naar methanogene bacteriën. Omdat in anaërobe ecosystemen en cocultures zéér lage $H_2$ en formiaat concentraties aanwezig zijn, is het niet bekend welke van de twee moleculen belangrijker is.

Het doel van dit onderzoek was om het relatieve belang van zowel $H_2$- als formiaatoverdracht op te helderen tijdens de syntrofe omzetting van propionaat en butyraat; hierbij gebruikmakend van een fysiologische en biochemische benadering.

Deze studie werd mogelijk nadat reincultures van de propionaatoxideerder MPOB en de butyraatoxideerder *Syntrophospora bryantii* waren verkregen door ze respectievelijk op fumaraat en crotonaat te kweken. Door gedefinieerde methanogene cocultures te maken, werd waargenomen dat propionaatoxidatie door MPOB en butyraatoxidatie door *S. bryantii* slechts dan optrad wanneer een $H_2$ + formiaat gebruikende methanogeen aanwezig was, en niet wanneer een $H_2$-gebruikende of aceticlastische methanogeen aanwezig was (Hoofdstuk 2 en 3). Dit kan er op duiden dat formiaatoverdracht overheerst in deze cultures. Evenwel, een triculture van MPOB en een *Desulfovibrio* stam, die formiaat in $H_2/CO_2$ omzet, samen met een $H_2$-gebruikende methanogeen kon wel propionaat omzetten. Dit bevestigt dat $H_2$-overdracht mogelijk is op voorwaarde dat de formiaatconcentraties laag worden gehouden (Hoofdstuk 2).

MPOB is in staat om fumaraat naar succinaat om te zetten én om propionaat-oxidatie aan fumaraatreductie te koppelen. *S. bryantii* is in staat om butyraatoxidatie te koppelen aan pentenoaat-reductie (Hoofdstuk 3). Met een beperkende hoeveelheid fumaraat in een propionaatoxiderende culture van MPOB konden maximale hoeveelheden van 6.8 Pa $H_2$ en 24 $\mu$M formiaat gevormd worden; met een beperkende hoeveelheid pentenoaat in een butyraatoxiderende culture van *S. bryantii* waren deze hoeveelheden respectievelijk 170 Pa voor $H_2$ en 280 $\mu$M voor formiaat (Hoofdstuk 4). Deze resultaten tonen aan dat zowel $H_2$ als formiaat daadwerkelijk gevormd worden en dat de waarden die gemeten worden binnen het bereik liggen dat verwacht mag worden op grond van thermodynamische berekeningen. Als een diffusiemodel gebruikt wordt, kan berekend worden dat formiaatfluxen 100 keer hoger zijn dan $H_2$-fluxen in de methanogene cocultures van MPOB en *S. bryantii*. Dit suggereert dat formiaatoverdracht belangrijker is dan $H_2$-overdracht tijdens syntrofe propionaat- en butyraatafbraak.

Uit enzymmetingen bleek (Hoofdstuk 5) dat *S. bryantii* een zeer hoge hydrogenase en een lage formiaatdehydrogenase activiteit bezat. De $K_m$-waarden van deze twee enzymen zijn ongeveer gelijk: respectievelijk 0.21 mM en 0.22 mM. Butyryl-CoA dehydrogenase en 3-hydroxybutyryl-CoA dehydrogenase, die betrokken zijn bij de twee oxidatie-reacties tijdens de butyraatoxydatie bleken oplosbare enzymen te zijn. Het hydrogenase bleek een cytoplasmatisch enzym te zijn, terwijl formiaatdehydrogenase en $CO_2$-reductase membraangebonden bleken, vermoedelijk gelokaliseerd aan de buitenkant van het cytoplasmatisch membraan. Deze waarnemingen suggereren dat $H_2$ intracellulair wordt gevormd tijdens de butyraatoxydatie, terwijl formiaat extracellulair gevormd wordt. De endergone oxidatie van butyryl-CoA naar crotonyl-CoA wordt verondersteld te worden gedreven door een omgekeerd elektronentransport mechanisme. Vergelijkbare experimenten werden gedaan met MPOB. Ook MPOB bevatte relatief hoge hydrogenase activiteiten (1.5-3.7 $\mu$mol.min$^{-1}$.mg$^{-1}$) en lage formiaatdehydrogenase activiteiten (0.2-0.4 $\mu$mol.min$^{-1}$.mg$^{-1}$). De $K_m$-waarden zijn 0.05 mM $H_2$ voor hydrogenase en 0.3 mM formiaat voor formiaatdehydrogenase. Na lokalisatie van de redoxenzymen die betrokken zijn bij de propionaatoxydatie bleek fumaraatreductase membraangebonden, vermoedelijk aan de binnenkant van de membraan, terwijl malaatdehydrogenase en pyruvaatdehydrogenase in het cytoplasma zaten. Het hydrogenase was gedeeltelijk in het periplasma, gedeeltelijk in het cytoplasma en gedeeltelijk in de membraan gelokaliseerd, terwijl formiaatdehydrogenase gedeeltelijk in het periplasma en gedeeltelijk in de membraan werd aangetroffen. Deze resultaten geven aan dat gedurende propionaatoxidatie, formiaat aan de buitenkant van de cytoplasmatische membraan wordt geproduceerd. Echter, waar $H_2$ wordt geproduceerd is nog niet duidelijk. Net als bij butyraatoxidatie, wordt verondersteld dat de oxydatie van succinaat ($\Delta G^{0\prime} = +86.2$ kJ/mol) wordt gedreven door een omgekeerd elekronentransport mechanisme.

Hoewel acetaat ook een eindprodukt van propionaat- en butyraatoxydatie is, leidde de verwijdering van acetaat door aceticlastische methanogenen alleen niet tot het laten verlopen van de oxydatiereacties door MPOB en *S. bryantii*. Echter, wanneer de aceticlastische methanogeen *Methanothrix (Methanosaeta) soehngenii* toegevoegd werd aan cocultures van MPOB en *S. bryantii* samen met een $H_2$ + formiaat gebruikende methanogeen, leidde dit wel tot verhoogde omzettingssnelheden van propionaat en butyraat (Hoofdstuk 2 en 3). Toevoeging van 50 mM acetaat aan een coculture van *S. bryantii* en *Methanospirillum hungatei* verminderde de butyraatomzettingssnelheid met 60% (Hoofdstuk 3). Deze resultaten geven aan dat ook een lage acetaatconcentratie bevorderlijk is voor de syntrofe omzetting van propionaat en butyraat.

# 小　结

大分子有机物矿化为甲烷和二氧化碳这一过程由发酵细菌，互营产乙酸细菌和产甲烷细菌共同完成．丙酸盐和丁酸盐是这个过程中两个重要的中间代谢产物，由它们降解所产生的甲烷占全部甲烷生成量的35%和6%．互营产乙酸细菌和产甲烷菌协同作用将丙酸盐和丁酸盐降解为甲烷和二氧化碳．种间分子氢转移和甲酸盐转移均被认为是这个互营降解的可能机制，可将互营产乙酸菌所产生的还原当量传递给产甲烷细菌．由于厌氧环境和共营培养物中的分子氢和甲酸盐水平都十分低，所以究竟哪一个更重要至今仍未知．

此项研究的目的在于通过生理和生化的手段去揭示分子氢转移和甲酸盐转移在丙酸和丁酸盐的互营降解中的相对重要性．

以延琥羧酸和巴豆酸为唯一碳源可分别获得丙酸盐氧化菌MPOB和丁酸盐降解菌-布氏互营生孢菌(Syntrophospora bryantii)的纯培养物，这使得此项研究得以进行．通过组成已知的产甲烷共培养物，发现只有既能利用氢又利用甲酸盐的产甲烷菌可以促使MPOB降解丙酸盐及促使布氏互营生孢菌降解丁酸盐，而只利用氢或只利用乙酸盐的产甲烷菌无此能力(第二章和第三章)，这说明甲酸盐转移可能在这些培养物中起主要作用．由MPOB和一个可转化甲酸盐为$H_2/CO_2$的脱硫弧菌(Desulfovibrio)菌株及一个只利用$H_2$的产甲烷菌的三元共培养物也可降解丙酸盐，进一步确证了只要保证很低的甲酸浓度，$H_2$转移才是可能发生的．

MPOB能够发酵延琥羧酸为琥珀酸，并能够将丙酸盐的氧化与延琥羧酸的还原相偶联．而布氏互营生孢菌能够将丁酸盐氧化与Pentenoate的还原相偶联(第三章)．在降解丙酸盐的MPOB培养物中仅加入一定限量的延琥羧酸，由丙酸盐降解所产生的$H_2$和甲酸盐的最大值分别为 6.8Pa 和 24umol；在降解丁酸盐的布氏互营生孢菌培养物中加入限量的pentenoate，由丁酸盐降解所产生的$H_2$和甲酸盐的最大值分别为 170Pa 和 280umol(第四章)．这些结果表明在丙酸盐和丁酸盐的降解中确实有$H_2$和甲酸盐产生，并且在热力学所允许的范围之内．用扩散模型计算得知在MPOB和布氏互营生孢菌的产甲烷共培养物中，甲酸盐的流量比$H_2$的流量大100倍，说明在丙酸盐和丁酸盐的互营降解中，甲酸盐转移更重要．

酶活测定(第五章)表明布氏互营生孢菌含有很高的氢化酶活力和低的甲酸脱氢酶活力．两个酶的$K_m$值基本相同，氢化酶为0.21mM的$H_2$，甲酸脱氢酶为0.22mM的甲酸盐．丁酰辅酶A脱氢酶和3-羟基丁酰辅酶A脱氢酶催化丁酸降解中的两个氧化反应，这两个酶存在于细胞质中．氢化酶也是细胞质中的酶，而甲酸脱氢酶和$CO_2$还原酶为膜蛋白酶，似乎位于细胞膜外侧．这些发现指出在丁酸盐降解中，$H_2$在细胞内形成而甲酸在细胞外产生．据推测一个电子反转移的机制用于推动丁酰辅酶A氧化为巴豆酰辅酶A这个吸能反应．类似的实验也在MPOB中进行(第六章)．MPOB也含有相对高的氢化酶活力(1.5-3.7umol/min/mg)和低的甲酸脱氢酶活力(0.2-0.4umol/min/mg)．氢化酶的$K_m$值为0.05mM的$H_2$，甲酸脱氢酶的$K_m$值为0.3mM的甲酸盐．对丙酸盐降解中的氧化还原酶的定位表明延琥羧酸还原酶位于细胞膜上，可能在膜的内侧，而苹果酸脱氢酶和丙酮酸脱氢酶位于细胞质中．氢化酶一部分位于细胞周质中，部分位于细胞质中，部分位于细胞膜上；而甲酸脱氢酶部分位于细胞周质中，部分位于外侧细胞膜上．这些结果表明在丙酸盐的降解中，甲酸盐在细胞膜外形成，然而分子氢的形成部位还未知，

与丁酸氧化相同，推测一个电子反转移机制驱动琥珀酸的氧化($\Delta G'$=86.2KJ/mol).

尽管乙酸盐也是丙酸盐和丁酸盐氧化的产物，但利用乙酸盐的产甲烷菌对乙酸盐的消耗不能驱动互营产乙酸细菌MPOB和布氏互营生孢菌的氧化反应．然而在MPOB及布氏互营生孢菌与$H_2$/甲酸盐营养型的产甲烷菌的互营培养物中加入利用乙酸盐的产甲烷菌宋氏甲烷丝菌(*Methanothrix soehngenii*)，可以增加其丙酸盐和丁酸盐的降解速率(第二章和第三章)．在布氏互营生孢菌和亨氏甲烷螺菌的互营培养物中加入50mM乙酸盐使其降解丁酸的速率降低了60%(第二章)．这些结果表明较低的乙酸盐浓度利于丙酸盐和丁酸盐的互营降解.

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# Curriculum vitae

The author of this thesis, Xiuzhu Dong was born in Hebei, China. She graduated from Agricultural University of Beijing, China in 1982, and obtained her master degree in Science at Institute of Microbiology, scientific Academy of China in 1984. The topic of the master thesis was on taxonomy of acetic acid bacteria. From 1985 till 1989, she was a research member of the department of bacteriology, Institute of Microbiology, Beijing. Since 1990, she was admitted to be a Ph.D. student through the "sandwich" program in the department of microbiology, Wageningen Agricultural University. She will return to China at the end of 1994 to continue the research in China.