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Metabolic interactions between anaerobic bacteria in methanogenic environments

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Abstract

In methanogenic environments organic matter is degraded by associations of fermenting, acetogenic and methanogenic bacteria. Hydrogen and formate consumption, and to some extent also acetate consumption, by methanogens affects the metabolism of the other bacteria. Product formation of fermenting bacteria is shifted to more oxidized products, while acetogenic bacteria are only able to metabolize compounds when methanogens consume hydrogen and formate efficiently. These types of metabolic interaction between anaerobic bacteria is due to the fact that the oxidation of NADH and $FADH_2$ coupled to proton or bicarbonate reduction is thermodynamically only feasible at low hydrogen and formate concentrations. Syntrophic relationships which depend on interspecies hydrogen or formate transfer were described for the degradation of e.g. fatty acids, amino acids and aromatic compounds.

Introduction

In mixed microbial communities microorganisms are constantly in interaction with each other. The nutritionally related interactions were divided into 6 types of metabolic interactions, neutralism, commensalism, mutualism, amensalism, predation or competition (Bull & Slater 1982). Solely the metabolic interactions between anaerobic bacteria in methanogenic environments are discussed here. Attention is focused on mutualistic interactions between fermenting, acetogenic and methanogenic bacteria caused by interspecies hydrogen and formate transfer. In addition, some competitive interactions between anaerobic bacteria in methanogenic environments are discussed. The metabolic interactions between methanogens and methanogenic associations and sulfate-reducing bacteria were described recently (Oude-Elferink et al. 1994).

Methanogenic environments

In methanogenic environments, organic compounds are degraded in the absence of inorganic electron acceptors (oxygen, nitrate, sulfate, sulfur or oxidized metal ions (Fe^{3+}; Mn^{4+}), i.e. only fermentation processes, and respiration processes with protons or bicarbonate as electron acceptors are possible. Examples of methanogenic habitats are freshwater environments (sediments, wetlands, swamps, paddy fields etc.), intestinal tracts of higher animals and insects, landfills and anoxic bioreactors (Oremland 1988; Boone 1991; Crill et al. 1991). In animals, organic intermediates of the anoxic digestion process can be absorbed in the blood and metabolized further in the animal tissue (Miller 1991). Fatty acids formed by fermenting bacteria in the rumen are considered as a major source of feed for the ruminants. In the other environments a complete mineralization of organic compounds to methane and carbon dioxide can be achieved.

Intriguing man-made methanogenic habitats are high-rate anoxic bioreactors. By process control,

methanogenesis has been optimized such that an efficient and complete removal of organic compounds is achieved within a relatively short period of time. Examples of high rate reactors are the Upflow Anaerobic Sludge Bed (UASB) reactor, the Fluidized Bed reactor and the Anaerobic Filter (Lettinga & Hulshoff Pol 1991; Iza 1991; Young 1991). The system with the widest application is the UASB reactor; at present more than 300 full-scale UASB reactors are in operation for the treatment of high strength industrial wastewaters (Fig. 1). The wastewater is pumped from the bottom into the UASB reactor, and purification takes place during the passage through a bed of methanogenic sludge. In this type of reactor self-immobilization of anaerobic bacteria into densely packed granular aggregates takes place (Fig. 2). In these methanogenic granules the different physiological types of bacteria are in close vicinity of each other, enhancing the mass transfer processes which play a key role in methanogenesis.

Anaerobic degradation under methanogenic conditions

Methanogenic bacteria are specialized in the break-down of a limited number of substrates. These include, H_2/CO_2, formate, acetate and a few other substrates like methanol, ethanol, isopropanol, methylated amines, methylated sulfur compounds, and pyruvate (Jones 1991; Vogels et al. 1988; Whitman et al. 1992). Some methanogens can only use one substrate, e.g. H_2/CO_2 is the only growth substrate for *Methanobrevibacter arboriphilus* (Zeikus & Henning 1975; Zehnder & Wuhrmann 1977), whereas *Methanothrix* (*Methanosaeta*) species metabolize only acetate (Huser et al. 1982; Patel 1984; Patel & Sprott 1990). Some other methanogens are somewhat more versatile; *Methanospirillum hungatei* and *Methanobacterium formicicum* grow on H_2/CO_2 and formate (Schauer et al. 1982; Bryant & Boone 1987), and *Methanosarcina* sp. use H_2/CO_2, acetate, methanol, methylated amines and pyruvate for growth (Whitman et al. 1992; Jones 1991). Due to this restricted metabolism of methanogens, organic compounds are degraded in methanogenic environments by associations of fermenting, acetogenic and methanogenic bacteria (Fig. 3). Complex organic molecules are fermented by a variety of fermenting organisms to compounds which can be used by methanogens directly (hydrogen, formate, acetate), and to reduced organic compounds (lactate, ethanol, propionate and butyrate), which have to be oxidized further by acetogenic bacteria to substrates which can be used by methanogens (Mah et al. 1990; Stams & Zehnder 1990; Gujer & Zehnder 1983). These latter anaerobic oxidation reactions which are carried out by acetogenic bacteria have a positive Gibbs free energy change ($\Delta G^{o\prime}$), and therefore they are possible only when the products are taken away by the methanogens. Table 1 summarizes some acetogenic reactions which have to be coupled to product removal by methanogens or other hydrogenotrophic anaerobes. Methanogens catabolize hydrogen, formate and acetate efficiently; the threshold values for hydrogen are generally below 10 Pa, whereas the lowest concentration of formate and acetate that can be reached is around 10 μM (Cord-Ruwisch et al. 1988; Schauer et al. 1982; Jetten et al. 1990). The need for product removal results in an obligately syntrophic growth of acetogenic and methanogenic bacteria. This syntrophic growth can be considered as growth at the absolute minimum of what is thermodynamically possible. Syntrophic associations like other chemotrophic bacteria derive metabolic energy from the Gibbs free energy change of chemical reactions. The synthesis of ATP from ADP and Pi requires about 32 kJ per mol (Thauer et al. 1977), but generally in biological systems an energy difference of about 60 to 70 kJ is required for the irreversible synthesis of 1 mol ATP, including heat losses. Thauer & Morris (1984) pointed out that during electron transport phosphorylation 3 protons are required for the synthesis of one ATP. Therefore, the minimum amount of energy which can support growth of microorganisms is thought to be about 20 kJ, the equivalent of 1 proton translocated across the charged cytoplasmic membrane. Some bacteria in syntrophic associations have to cope with this minimum amount of energy.

General mechanism of interspecies electron transfer

Some fermenting bacteria and acetogenic bacteria have the ability to dispose of reducing equivalents by the reduction of protons or bicarbonate. Different types of electron mediators may be involved in the break-down of substrates. NAD(P) is a common electron acceptor in the anaerobic degradation of sugars (glyceraldehyde-3P dehydrogenase), amino acids (e.g. alanine and glutamate dehydrogenase) and organic acids (lactate dehydrogenase, malate dehydrogenase). Ferredoxin (Fd) is involved in some oxidative decarboxylations, e.g. pyruvate and α-ketoglutarate oxi-

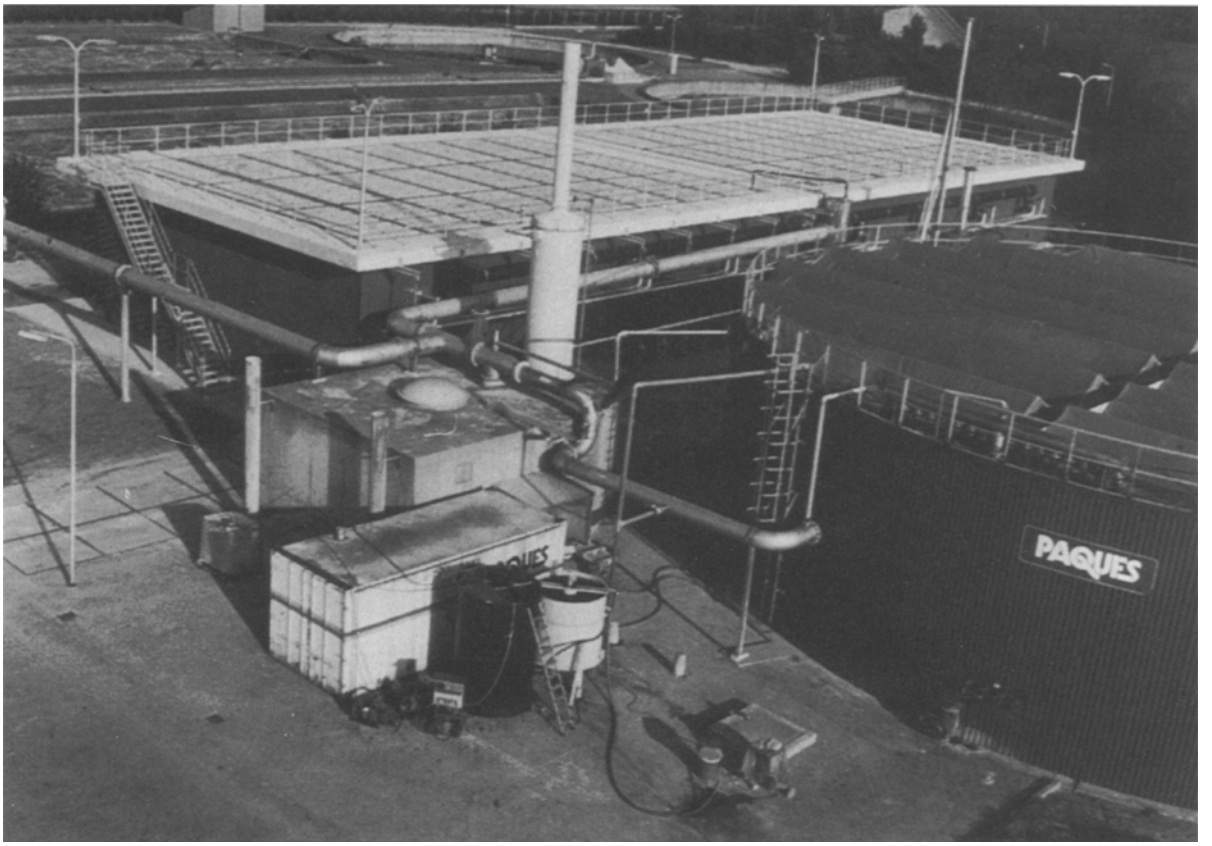

Fig. 1. Purification plant near Eerbeek (The Netherlands) treating wastewater from three paper mills, showing an old type of UASB reactor type (right) and the new UASB reactor type which consists of separate modules (left).

dation to acetyl-CoA and succinyl-CoA, respectively. FAD is involved in some special dehydrogenation reactions of fatty acid-oxidizing acetogens. The redox couples of $Fd_{(ox)}/Fd_{(red)}$, NAD/NADH and FAD/$FADH_2$ are −398, −320, and −220 mV, respectively, whereas the redox couples H^+/H_2 and HCO_3^-/formate are −414 and −407 mV, respectively (Thauer et al. 1977). The Gibbs free energy changes ($\Delta G^{\circ\prime}$ values) of redox reactions can be calculated using $\Delta G^{\circ\prime} = -n.F.\Delta E^{\circ\prime}$, where n is the number of electrons transferred, F is the Faraday constant and $\Delta E^{\circ\prime}$ is the difference between the redox couples:

	$\Delta G^{\circ\prime}$
$2\ Fd_{(red)} + 2H^+ \rightarrow 2\ Fd_{(ox)} + H_2$	+ 3.1 kJ
$2\ Fd_{(red)} + HCO_3^- \rightarrow 2\ Fd_{(ox)} + Formate^- + H^+$	+ 1.7 kJ
$NADH + H^+ \rightarrow NAD^+ + H_2$	+ 18.1 kJ
$NADH + HCO_3^- + H^+ \rightarrow NAD^+ + Formate^- + H_2O$	+ 16.8 kJ
$FADH_2 \rightarrow FAD + H_2$	+ 37.4 kJ
$FADH_2 + HCO_3^- \rightarrow FAD + Formate^- + H_2O$	+ 36.1 kJ

NADH and $FADH_2$ oxidation coupled to proton or bicarbonate reduction is difficult because of the positive $\Delta G^{\circ\prime}$values. However, $\Delta G^{\circ\prime}$ values represent Gibbs free energy changes at standard conditions, 298°K, pH 7, 1M for solutes and 10^5 Pa (1 atm) for gases. In biological systems the concentrations are much lower. Hydrogen and formate formation becomes more favorable if the concentration of these compounds is kept low by methanogens. E.g. the $\Delta G'$ of NADH oxidation coupled to proton reduction is given by:

$$\Delta G' = \Delta G^{\circ\prime} + RTln\frac{[NAD^+].P_{H2}}{[NADH].[H^+]} \qquad (1)$$

The effect of the hydrogen partial pressure on hydrogen formation from the different redox mediators is given in Fig. 4. At a hydrogen partial pressure of 10 Pa (10^{-4} atm) NADH oxidation coupled to hydrogen formation is energetically feasible. However, the oxidation of $FADH_2$ to proton reduction requires far low-

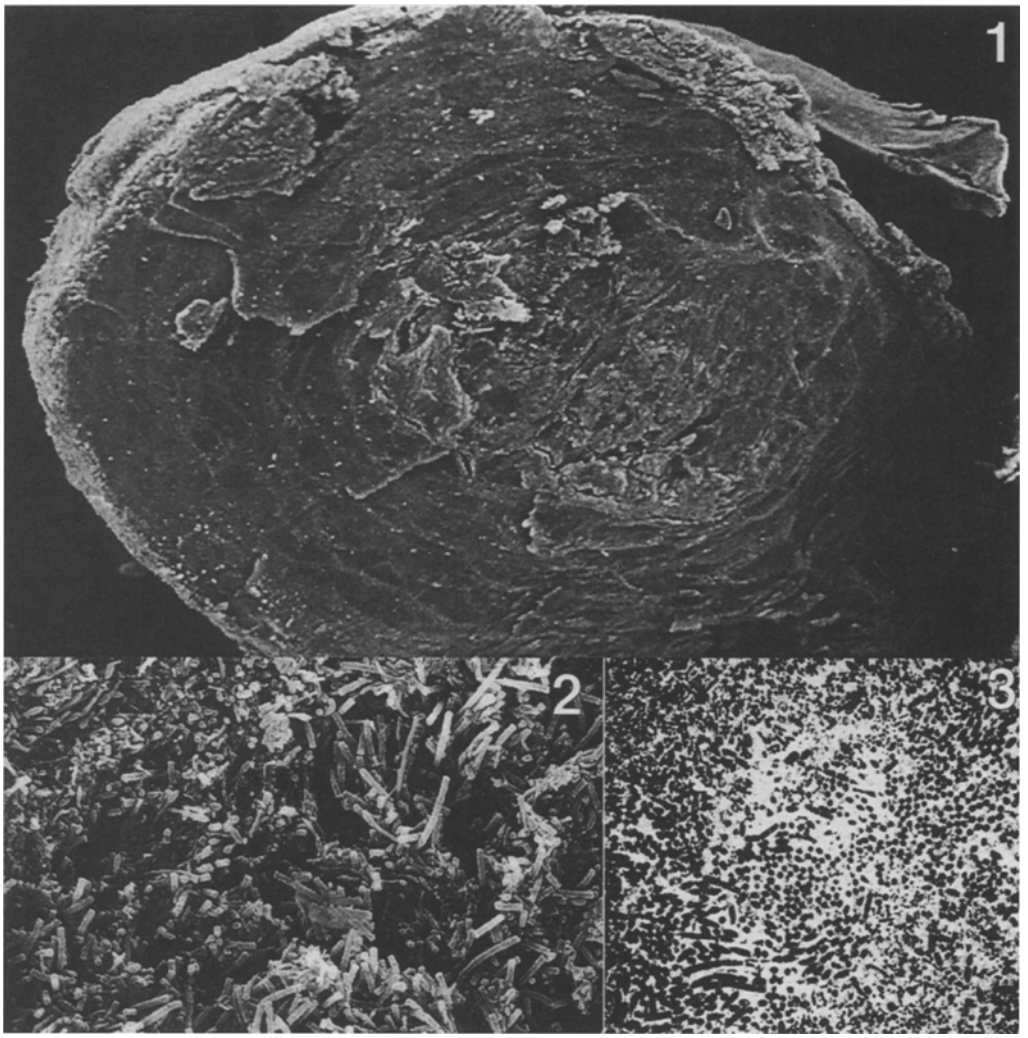

Fig. 2. Micrographs of methanogenic granular sludge. 1) cross section of a methanogenic granule of about 3 mm in size, 2) and 3) scanning and transmission electron micrographs showing the high biomass density of the granules.

er hydrogen partial pressures than can be achieved by methanogens. Therefore, acetogenic bacteria which form $FADH_2$ in catabolic processes have to invest metabolic energy to 'pull' this oxidation. A proton motive force-driven reversed electron flow has to be proposed. Evidence for a reversed electron flow-mediated hydrogen formation was obtained for syntrophic degradation of glycolate (Friedrich et al. 1991; Friedrich & Schink 1993).

Facultatively syntrophic sugar-fermenting associations

The classical example of the effect of interspecies electron transfer on sugar fermentation is *Ruminococcus albus* growing in the presence and absence of *Wolinella* (former *Vibrio*) *succinogenes* (Ianotti et al. 1973). In pure culture this organism forms acetate, CO_2, hydrogen and ethanol as end products, while in the coculture ethanol is not formed (Fig. 5). *R. albus* degrades glucose via glycolysis. This results in the formation of NADH (glyceraldehyde-3-phosphate dehydrogenase) and reduced ferredoxin (pyruvate:ferredoxin oxidoreductase). The oxidation of reduced ferredoxin is easily

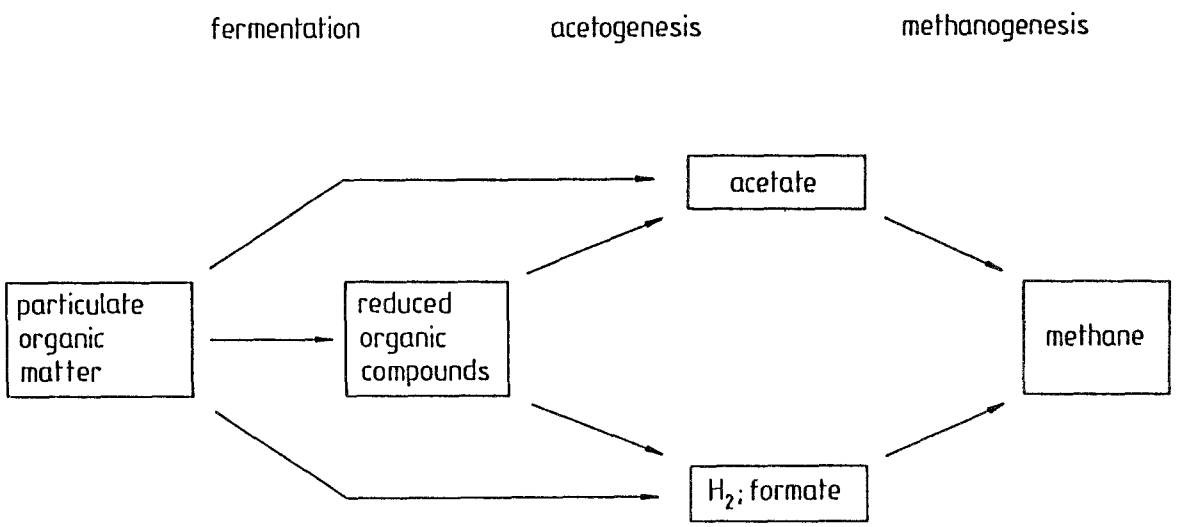

Fig. 3. Degradation of organic matter under methanogenic conditions, involving fermenting, acetogenic and methanogenic bacteria.

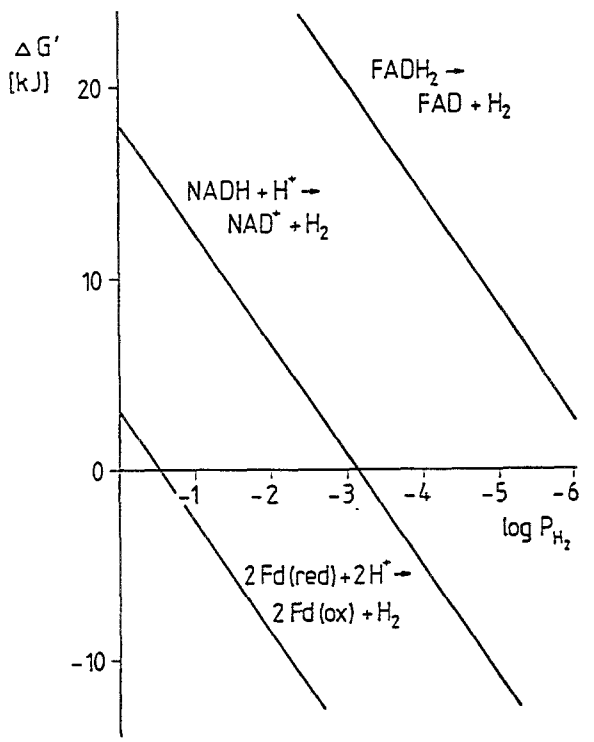

Fig. 4. Gibbs free energy changes of electron carrier oxidations at different hydrogen partial pressures.

coupled to hydrogen formation. However, H_2 formation from NADH is not possible at a high hydrogen partial pressure, and therefore, the organism couples the oxidation of NADH to the reduction of acetyl-CoA to ethanol. In the coculture hydrogen is removed efficiently, and ethanol is no longer produced. Because the organism forms ATP during acetate formation from acetyl-CoA, but not if ethanol is produced from acetyl-CoA, the ATP yield per glucose is higher in the coculture than in the pure culture.

Similar effects have been described for *Ruminococcus flavefaciens* (Latham & Wolin 1977), *Selenomonas ruminantium* (Scheifinger et al. 1975; Chen & Wolin 1977), *Clostridium cellobioparum* (Chung 1976), *Clostridium thermocellum* (Weimer & Zeikus 1977), *Bacteroides xylanolyticus* (Scholten Koerselman et al. 1986; Biesterveld & Stams 1990; Biesterveld et al. 1994), *Acetobacterium woodii* (Winter & Wolfe 1980), a homoacetogenic bacterium EE121 (Plugge et al. 1990) and anaerobic fungi (Marvin-Sikkema et al. 1990). *Acetobacterium woodii* and strain EE121 are able to dispose off reducing equivalents by the reduction of CO_2 to acetate; in pure culture 1 mol of sugar is fermented to 3 mol of acetate. Such homoacetogens have a poor affinity for hydrogen (Cord-Ruwisch et al. 1988; Table 3). Therefore, in coculture with methanogens these bacteria ferment sugars to 2 acetate, 2 CO_2 and hydrogen, the latter being consumed by the methanogen.

We have studied the effect of the hydrogen partial pressure on xylose metabolism of *Bacteroides xylanolyticus*. This bacterium degrades glucose via glycolysis and xylose via the pentose phosphate pathway in junction with glycolysis (Biesterveld et al.

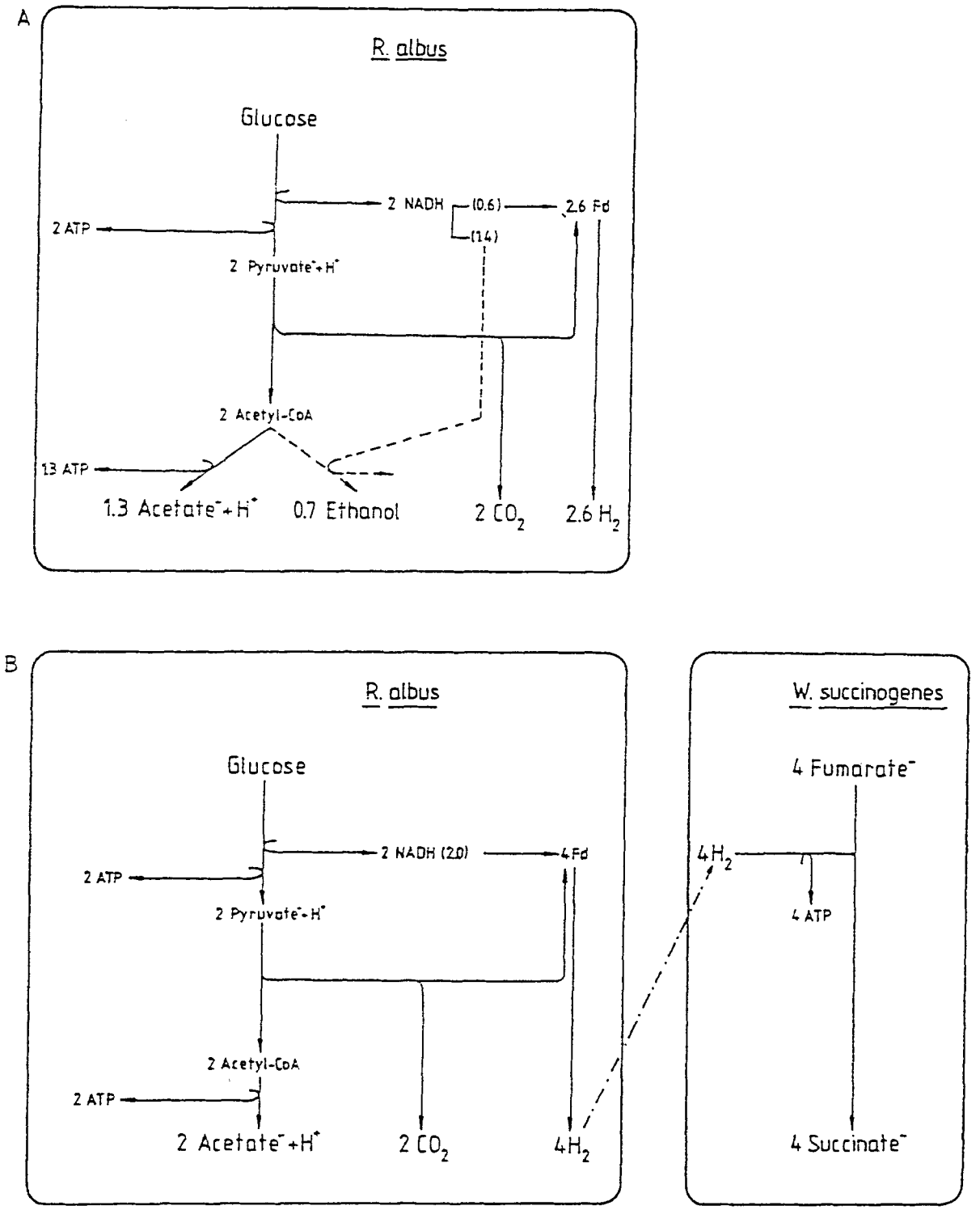

Fig. 5. Sugar fermentation by *Ruminococcus albus* in the absence and the presence of a hydrogen-consuming anaerobe (Ianotti et al. 1973).

Table 1. Some reactions involved in syntrophic degradation processes

	$\Delta G^{\circ\prime}$ [kJ/reaction]
Acetogenic reactions[a]	
Ethanol and lactate	
$Ethanol + H_2O \rightarrow Acetate^- + H^+ + 2\ H_2$	+ 9.6
$Lactate^- + 2\ H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 2\ H_2$	− 4.2
Fatty acids	
$Acetate^- + 4\ H_2O \rightarrow 2\ HCO_3^- + H^+ + 4\ H_2$	+104.6
$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$	+ 76.1
$Butyrate^- + 2H_2O \rightarrow 2\ Acetate^- + H^+ + 2H_2$	+ 48.1
$Caproate^- + 3\ H_2O \rightarrow 3\ acetate^- + 2\ H^+ + 4\ H_2$	+ 96.2
Amino acids	
$Alanine + 3\ H_2O \rightarrow acetate^- + HCO_3^- + NH_4^+ + H^+ + 2H_2$	+ 7.5
$Aspartate^- + 4H_2O \rightarrow acetate^- + 2\ HCO_3^- + NH_4^+ + H^+ + 2\ H_2$	− 14.0
$Leucine + 3\ H_2O \rightarrow isovalerate^- + HCO_3^- + NH_4^+ + H^+ + 2\ H_2$	+ 4.2
$Glutamate^- + 4\ H_2O \rightarrow propionate^- + 2\ HCO_3^- + NH_4^+ + H^+ + 2H_2$	− 5.8
$Glutamate^- + 7\ H_2O \rightarrow acetate^- + 3\ HCO_3^- + 3\ H^+ + 5H_2$	+ 70.3
Aromatic compounds	
$Benzoate^- + 7\ H_2O \rightarrow 3\ acetate^- + HCO_3^- + 3\ H^+ + 3\ H_2$	+ 58.9
$Hydroxybenzoate^- + 6\ H_2O \rightarrow 3\ acetate^- + HCO_3^- + 3\ H^+ + 2\ H_2$	+ 5.2
$Phenol + 5\ H_2O \rightarrow 3\ acetate^- + 3\ H^+ + 2\ H_2$	+ 5.5
Hydrogenotrophic reactions[a]:	
$4H_2 + 2\ HCO_3^- + H^+ \rightarrow acetate^- + 4\ H_2O$	−104.6
$4\ H_2 + 4\ S^\circ \rightarrow 4\ HS^- + 4H^+$	−112
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	−135.6
$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\ H_2O$	−151.9
$4\ H_2 + 4\ fumarate^{2-} \rightarrow 4\ succinate^{2-}$	−344.6
$4\ H_2 + NO_3^- + 2\ H^+ \rightarrow NH_4^+ + 3\ H_2O$	−599.6
Formate-hydrogen interconversion[b])	
$H_2 + HCO_3^- \rightarrow Formate^- + H_2O$	− 1.3
Acetate cleavage	
$Acetate^- + H_2O \rightarrow HCO_3^- + CH_4$	− 31.0

[a] In methanogenic environments the acetogenic reactions are coupled to methanogenesis from H_2/CO_2 and from acetate.

[b] In case of formate transfer, the $\Delta G^{\circ\prime}$ values can be calculated by combining this reaction with the acetogenic reactions and hydrogenotrophic reactions.

1994a). Like in *R. albus*, NAD^+ and ferredoxin are the intracellular redox mediators, and product formation is affected in a similar way as described above for *R. albus*. The flow of electrons in *B. xylanolyticus* was determined in xylose-limited chemostat cultures in the presence or the absence of *Methanospirillum hungatei* (Biesterveld et al. 1990; 1994b). At steady state conditions the product formation and enzyme levels in cell extracts were analyzed. In the coculture ethanol was not produced, and ethanol dehydrogenase and acetaldehyde dehydrogenase were not detected. When the methanogens were inhibited by bromo-ethane sulfonate (BrES) these enzymes were rapidly induced, and H_2, ethanol and formate were formed. When chloramphenicol, an inhibitor of protein synthesis, was added together with BrES xylose was only isomerized to xylulose but not degraded further (Biesterveld et al. 1994b). These results indicate that the shift in product formation is not only determined by a change in the flux through the different pathways, but that a regulation at the level of enzyme synthesis occurs as well. Recently the regulation of product formation of the anaerobic fungus *Neocallimastix* in pure culture and in coculture with methanogens was studied

Table 2. Measured highest hydrogen and formate concentrations that can be reached by acetogenic bacteria

Reaction	Organism	Temp [°C]	Hydrogen [Pa]	Formate [μM]	Reference
Ethanol oxidation	*P. acetylenicus*	28–34	3200		Seitz et al. 1988
Lactate oxidation	*Desulfovibrio*	28–34	4200		Seitz et al. 1988
Propionate oxidation	*S. wolinii*	37	± 10		Stams unpublished
	MPOB	37	13–16		Dong unpublished
	MPOB	37		40	Dong unpublished
	TPOB	55	30–34		Stams et al. 1992
Butyrate oxidation	*S. wolfei*	37	38		Boone et al. 1989
	S. bryantii	37	30–300		Dong unpublished
	S. bryantii	37		300	Dong unpublished
	NSF-2	37	95		Dwyer et al. 1988
	enrichment	55	300		Ahring & Westermann 1987b
Acetate oxidation	*'Reversibacter'*	60	60		Lee & Zinder 1988a, 1988b

Table 3. Threshold values for hydrogen and formate of different anaerobic bacteria

Reaction	Organism	Temp [°C]	Hydrogen [Pa]	Formate [μM]	Reference
Acetogenesis	*A. woodii*	28–34	52		Cord-Ruwisch et al. 1988
	A. carbinolicum	28–34	95		Cord-Ruwisch et al. 1988
	A. kivui	60	500		Zinder 1994
methanogenesis	*M. hungatei*	28–34	2.5		Seitz et al. 1988
	M. hungatei	28–34	3		Cord-Ruwisch et al. 1988
	M. hungatei	37		15	Schauer et al. 1982
	M. hungatei	37	16		Dwyer et al. 1988
	M. formicicum	37	5.9		Boone et al. 1989
	M. formicicum	28–34	2.8		Cord-Ruwisch et al. 1988
	M. formicicum	37		26	Schauer et al. 1982
	M. arboriphilus	28–34	9		Cord-Ruwisch et al. 1988
	M. thermoformicicum	55	6–11		Stams et al. 1992
	M. thermoformicicum	60	12		Zinder 1994
	M. thermoautotrophicum	55	6–11		Stams et al. 1992
	M. jannaschii	85	50–100		Kengen, unpublished
Sulfate reduction	*Desulfovibrio*	37	4.5		Boone & Bryant 1980
	Desulfovibrio	28–34	0.9		Cord-Ruwisch et al. 1988
	Desulfovibrio	37	3		Dwyer et al. 1988
Fumarate reduction	*W. succinogenes*	28–34	0.002		Cord-Ruwisch et al. 1988
Sulfur reduction	*W. succinogenes*	28–34	0.5		Cord-Ruwisch et al. 1988
Nitrate reduction	*W. succinogenes*	28–34	0.002		Cord-Ruwisch et al. 1988

(Marvin-Sikkema et al. 1993). Like in *B. xylanolyticus* enzymes involved in the formation of reduced products (fumarate reductase, ethanol dehydrogenase, acetaldehyde dehydrogenase and lactate dehydrogenase) were completely repressed.

Reducing equivalents in the form of NADH are thought to be transferred to hydrogen via ferredoxin

(Thauer et al. 1977). For this a NADH:ferredoxin oxidoreductase is required. This enzyme has been demonstrated in some anaerobic bacteria and in *Neocallimastix.* However, it was not detected in *Clostridium thermocellum* (Lamed & Zeikus 1980) and in *B. xylanolyticus.* The flow of electrons in these organisms is not clear yet. In *B. xylanolyticus* very high activities of an NADPH:ferredoxin oxidoreductase were demonstrated, but the function of this enzyme is not understood, as the glyceraldehyde-3-phosphate dehydrogenase is strictly NAD-dependent and a NADPH:NAD^+ oxidoreductase (transhydrogenase) could not be detected (Biesterveld et al. 1994a; 1994b). For *C. thermocellum* it has been assumed that a transhydrogenation may occur via an interconversion of malate, oxaloacetate and pyruvate involving a NADP-dependent malic enzyme, an oxaloacetate decarboxylase and a NAD-dependent malate dehydrogenase (Lamed & Zeikus 1980).

Although the oxidation of glucose to 2 acetate, 2 CO_2 and 4 H_2 is exergonic ($\Delta G^{\circ\prime} = -206.3$ kJ/mol) no mesophilic organisms have been described thus far which can perform this conversion in pure culture. Reduced organic compounds (ethanol, lactate, propionate or butyrate) are always produced in order to regenerate NAD. Krumholz & Bryant (1986) isolated *Syntrophococcus sucromutans* which degraded sugars, but was not able to from reduced organic products. This organism was obligately dependent on methanogens or the presence of an external electron acceptor. Moderately thermophilic bacteria have been described which are able to convert sugars to 2 acetate, 2 CO_2 and 4 H_2 (Dietrich et al. 1988; Soutschek et al. 1984; Cheng et al. 1992). Hydrogen formation at higher temperature is energetically more feasible than at lower temperatures.

Obligate syntrophic associations

Obligately syntrophic degradations have been described for a number of compounds (McInerney 1988; Dolfing 1988; Stams & Zehnder 1990; Schink 1992). These include alcohols (ethanol, methanol), fatty acids (acetate, propionate, butyrate, higher fatty acids and branched chain fatty acids like isobutyrate and isovalerate), amino acids (glutamate, aspartate, alanine, valine, histidine, leucine, isoleucine), other organic acids (lactate, malate, glycolate) and aromatic compounds (benzoate, hydroquinones, phenol). At least one oxidation step in the conversion of these compounds is energetically difficult, and has to be 'pulled' by methanogens or other hydrogen/formate-utilizing anaerobes. Thus, low concentrations of hydrogen and formate are essential. Moreover, the rate of conversion is determined by the flux of hydrogen and formate in the system. The latter is of particular importance because the concentration gradients between the acetogenic and methanogenic bacteria can be rather flat. Some factors which determine the flux of hydrogen and formate between acetogenic and methanogenic bacteria are discussed.

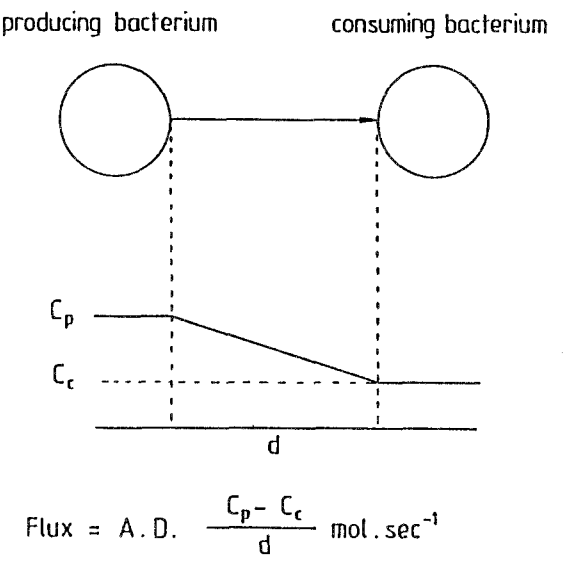

Fig. 6. Mass transfer by diffusion between a producing and a consuming organism. The flux is determined by the surface area (A) of the producing bacterium, the diffusion coefficient (D), the concentration at the surface of the producing and consuming organism (C_p and C_c, respectively) and the interbacterial distance (d).

Flux of formate and hydrogen

During interspecies electron transfer, hydrogen and formate have to diffuse from the producing organism to the consuming organism. The flux of hydrogen and formate can be described by a simple diffusion equation (Schink & Thauer, 1988). The flux of hydrogen or formate is directly dependent on the surface area of the producing bacterium, the diffusion constant of hydrogen or formate, the concentration difference between the producing and the consuming organism, and the distance between the two organisms (Fig. 6).

Concentration gradient. The maximum difference in concentration between the producing and the consuming bacterium is determined by the thermodynamical borders of the conversions carried out by these organisms. These thermodynamical borders refer to non-growing conditions; if the organisms have to conserve metabolic energy for growth, the difference in concentration is smaller. The highest concentration that can be formed by the producing organism and the lowest concentration that can be reached by the consuming organism can be calculated from data in Table 1, provided that the concentrations of other compounds are known. Such calculations were made for the degradation of a number of compounds in different anoxic environments (Conrad et al. 1986). Cord-Ruwisch et al. (1988) showed that the measured threshold values for hydrogen of the different types of hydrogenotrophic bacteria correlated with values expected from the $\Delta G^{\circ\prime}$ values. The threshold value for hydrogen of methanogens and sulfate reducers are 3–10 and 1–2 Pa, respectively (Table 3). These differences may explain why the growth rate of acetogenic bacteria depends on the type of hydrogenotrophic organism. 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 coculture with sulfate reducers these values were 0.19, 0.31, and 0.13 day^{-1}, respectively (Boone & Bryant, 1980; McInerney et al. 1979; Mountfort & Bryant 1982).

Bacterial distances. The diffusion distance between producing and consuming organism is largely determined by the biomass density. The clustering of cells will lead to increased fluxes (Schink & Thauer 1988). Assuming that bacteria have a diameter of 2 μm, it can be calculated that in cultures containing 10^8, 10^9, 10^{10} and 10^{11} cells per ml the average interbacterial distances are about 25, 10, 4 and 0.5 μm, respectively. Therefore, in a syntrophic type of degradation the activity per cell, and consequently the specific growth rates of cells are not constant but increase with decreasing interbacterial distances. This effect may explain the apparently long lag-phases which are often observed during subcultivation of syntrophic cultures. In methanogenic granular sludge the cell densities are extremely high. Values were reported which are close to what is theoretically possible (Grotenhuis et al. 1991; Dubourguier et al. 1988; Dolfing et al. 1985). Such high cell densities are favorable for interspecies electron transfer, resulting in very high methanogenic activities with propionate and butyrate as substrates. In propionate-adapted methanogenic granules, microbial structures were observed in which the distances between propionate-oxidizing bacteria and methanogens were within the μm range (Grotenhuis et al. 1991). Disrupture of the structure led to a reduction of the propionate degradation rate by 90 percent, while the acetate degradation rate by methanogens was largely unaffected. Similar observations were also made for the degradation of butyrate and propionate by thermophilic granules (Schmidt & Ahring 1993). Moreover, shortening of interbacterial distances by the creation of artificial precipitates (Stams et al. 1992) or by the addition of extra methanogens (Dwyer et al. 1988; Schmidt & Ahring 1993) led to increased methane formation rates with propionate and butyrate.

Temperature effect. Using the van 't Hoff equation Gibbs free energy changes at different temperatures can be calculated. ΔG values and ΔH values have been listed by Thauer et al. (1977) and Chang (1977), respectively. A change in temperature has an effect on the reaction-dependent part and on the concentration-dependent part of the $\Delta G'$ values. Calculations of $\Delta G'$ values at different temperatures show that hydrogen formation becomes energetically more favorable at higher temperatures, whereas hydrogen-consuming reactions become less favorable. This is illustrated in Fig. 7 where as examples the calculated temperature dependence of methane formation from $H_2 + CO_2$ and the oxidation of acetate to CO_2 and H_2 are given. Thermodynamically, lower hydrogen partial pressures can be reached by the methanogens at lower temperatures. The opposite is true for the highest hydrogen concentrations that can be formed by the acetogens. These calculations fit with the lowest and highest hydrogen partial pressures which have been measured with mesophilic and thermophilic methanogens and acetogens (Tables 2 and 3). A similar but somewhat lower effect can be calculated for formate consumption by methanogens and formate formation by acetogens. An increase in temperature affects the flux of hydrogen and formate in two ways: i) the diffusion coefficients of these compounds become higher and ii) the concentration gradient between the methanogen and the acetogen becomes steeper. Some examples of the effect of temperature on syntrophic degradation are given below.

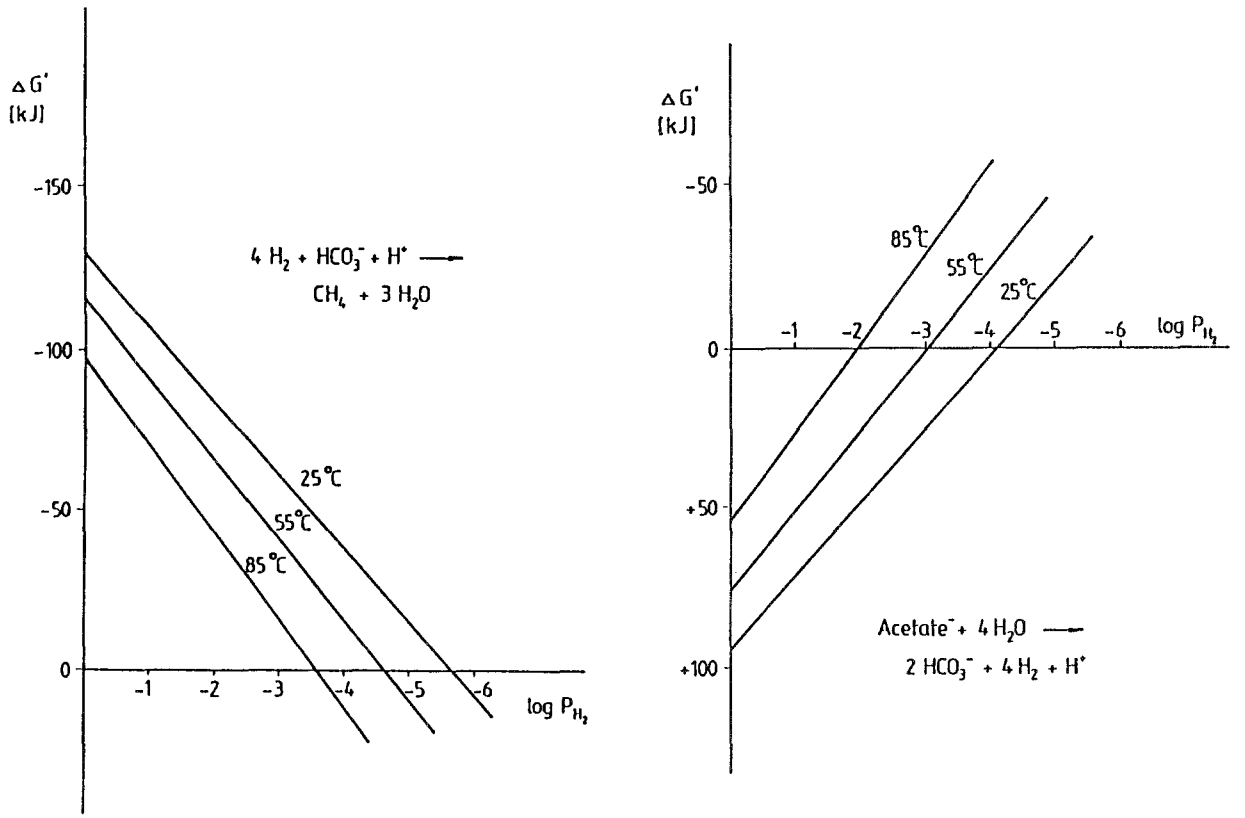

Fig. 7. Temperature dependence of hydrogen consumption by methanogens and acetate oxidation by acetogens. $\Delta G^{\circ\prime}$ values were calculated for solute concentrations of 20 mM.

Relative importance of hydrogen and formate

In the first papers on the syntrophic degradation by cocultures both interspecies hydrogen and formate transfer were proposed as possible mechanisms (Bryant et al. 1967; McInerney et al. 1979; Boone & Bryant 1980). Because in syntrophic cultures both the hydrogen and the formate concentration are extremely low, it is difficult to deduce which of the two is most important. This particularly because: i) the methanogens or sulfate reducers present in syntrophic cultures often can use both hydrogen and formate, ii) many acetogens are able to form both hydrogen and formate, and iii) methanogens and sulfate reducers are able to interconvert formate and hydrogen (Wu et al. 1993; Guyot & Brauman, 1986). In many studies concerning interspecies electron transfer methanogens could be used which only oxidized hydrogen (Bryant et al. 1967; McInerney et al. 1981; Ahring & Westermann 1987a,b; Lee & Zinder 1988a, Stams & Hansen 1984; Stams et al. 1992). However, the occurrence of formate transfer was clearly demonstrated in cocultures with *Desulfovibrio baarsii*, an organism that can oxidize formate but not hydrogen (Zindel et al. 1988). For a long period of time interspecies hydrogen transfer as formulated by Wolin (1976; 1982) was accepted as the most important mechanism by which electrons are shuttled in syntrophic cocultures.

First experimental evidence that formate transfer might be an additional or even a more important mechanism than hydrogen transfer was obtained with bacterial flocs from a reactor treating whey (Thiele & Zeikus 1987,1988; Thiele et al. 1988). Boone et al. (1989) calculated that in their butyrate-degrading cultures the measured rate of methane formation could not be explained with hydrogen transfer. Using diffusion models they showed that interspecies formate transfer should be about 100 times more important than interspecies hydrogen transfer. Their calculations were largely based on the differences in diffusion coefficients and solubilities of the two compounds. Hydrogen is poorly soluble in water; at a

hydrogen partial pressure of 10^5 Pa the concentration is only around 1 mM. Therefore, the concentration difference between the acetogens and the methanogens can be more than 1000 times higher with formate than with hydrogen, while the diffusion constants of hydrogen and formate in water differ only 30 times, 0.045 and 0.0015 mm^2s^{-1}, respectively. As a consequence, the flux of formate can be much higher than the flux of hydrogen. However, Schink (1992) hypothesized that during syntrophic degradation formate and hydrogen have to pass two lipophilic membranes, and that hydrogen but not formate may easily diffuse across lipophilic membranes. Recent observations made at our laboratory with defined butyrate- and propionate-degrading suspended cocultures indicate that low formate concentrations are essential. *Syntrophospora bryantii* degraded butyrate and a newly enriched acetogenic bacterium (MPOB) degraded propionate in coculture with *Methanospirillum hungatei* or in coculture with *Methanobacterium formicicum*, but not in coculture with two non-formate-utilizing *Methanobrevibacter* strains, unless a bacterium was present which was able to interconvert formate to H_2 + CO_2 (Dong, unpublished results). These findings suggest that hydrogen transfer could occur, albeit at a lower rate, provided that the concentration of formate is low. The rates of propionate and butyrate degradation were higher in cocultures with *M. hungatei* than in cocultures with *M. formicicum.* These differences can be explained if formate transfer is important. *M. hungatei* and *M. formicicum* have different threshold values of formate (15 and 26 μM, respectively), while the threshold values of hydrogen are about the same (Table 3).

In propionate-degrading granular sludge, *Methanobrevibacter* was the most numerous methanogen, and the rate of methane formation could be explained by interspecies hydrogen transfer (Grotenhuis et al. 1991; Stams et al. 1989). Remarkably, in this propionate-adapted sludge only low numbers of propionate-degrading acetogenic bacteria were counted with the most probable number technique, despite the fact that the propionate degradation rate of the sludge was very high (Grotenhuis et al. 1991). This observation can be explained if in suspended cultures formate transfer is more important than hydrogen transfer; for in this sludge *Methanobrevibacter* was the most abundant methanogen. In later studies we were able to count much higher numbers of acetogenic bacteria after the tubes were inoculated with *Methanobacterium formicicum* (Visser et al. 1994). In methanogenic granular sludge, interspecies hydrogen transfer might be most important, possibly because at short interbacterial distances the easy diffusibility of hydrogen across the lipophilic membranes compensates for the flat gradient of hydrogen between the acetogen and the methanogen.

Syntrophic degradation of lactate and ethanol

The existence of obligately syntrophic degradation by interspecies electron transfer was recognized for the first time by Bryant et al. (1967). It was observed that the originally believed pure culture of the ethanol-degrading *Methanobacillus omelianskii* consisted of two bacterial species, the S-organism which degraded ethanol to acetate and the methanogen strain M.o.H. Several other organisms were described since, including *Pelobacter* species (Schink 1984, 1985; Schink & Stieb 1983, Dubourguier et al. 1986), homoacetogens (Winter & Wolfe 1980; Plugge et al. 1990), *Thermoanaerobium brockii* (Ben-Bassat et al. 1981) and *Desulfovibrio* sp. (Bryant et al. 1967; Kremer et al. 1988) which are able to couple ethanol oxidation to methanogenesis. *Desulfovibrio* sp. are also able to oxidize lactate to acetate when cocultured with methanogens. In *Desulfovibrio* lactate oxidation but not ethanol oxidation is coupled to growth (Kremer et al. 1988). This is due to the fact that acetaldehyde is not oxidized via acetyl-CoA to acetate. However, in *Pelobacter acetylenicus* acetyl-CoA is an intermediate in ethanol oxidation (Fig. 8). Based on growth yield data obtained by Seitz et al. (1988), Schink (1992) concluded that *P. acetylenicus* has to invest part of this energy to pull the oxidation of ethanol, probably by an energy dependent acetate excretion.

Propionate oxidation

The complete mineralization of propionate requires three different bacteria, one acetogenic bacterium, a hydrogenotrophic methanogen and an aceticlastic methanogen (Table 1). Boone & Bryant (1980) described *Syntrophobacter wolinii* in a defined coculture with *Desulfovibrio* G11. Only recently a binary coculture of *S. wolinii* and *M. hungatei* was obtained (Dörner 1992). Some other mesophilic and thermophilic cultures were described which degrade propionate to acetate (Boone et al. 1989; Dörner 1992; Koch et al. 1983; Mah et al. 1988; Mucha et al. 1988; Stams

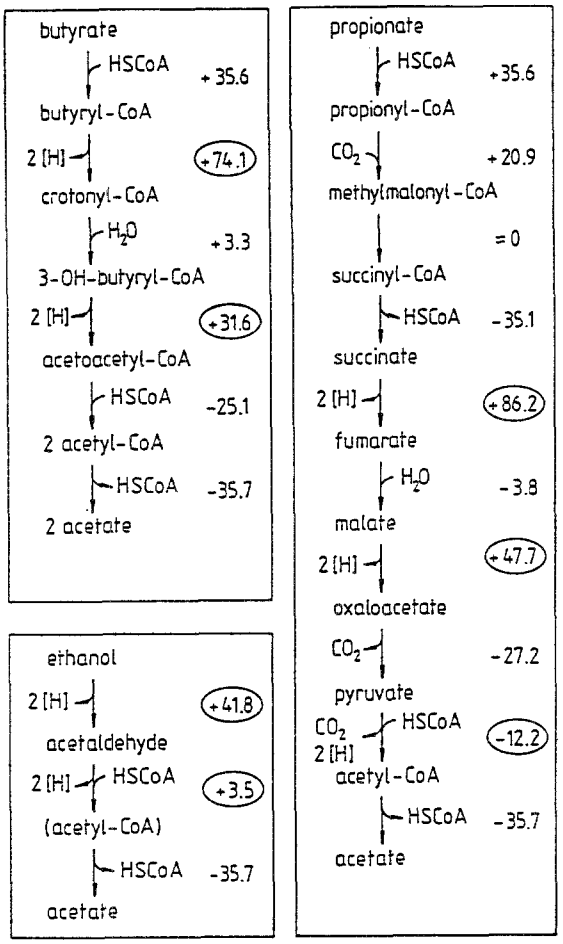

Fig. 8. Biochemical pathways of syntrophic ethanol, propionate and butyrate oxidation. The numbers refer to the $\Delta G^{\circ\prime}$ values of the different intermediate steps; the oxidation reactions are coupled to hydrogen formation.

et al. 1992, 1993). Tholozan et al. (1988) enriched a culture in which part of the propionate was degraded via a reductive carboxylation to butyrate.

Experiments with ^{13}C and ^{14}C labelled propionate (Koch et al. 1983; Robbins, 1988, Houwen et al. 1987, 1991), and enzyme measurements in cell extracts (Houwen et al. 1990; Plugge et al. 1993) have indicated that the methylmalonyl-CoA pathway is involved in syntrophic propionate oxidation (Fig. 8). A similar pathway was found in sulfate dependent propionate oxidation by *Desulfobulbus propionicus* (Stams et al. 1984 ; Kremer & Hansen 1988). In *S. wolinii* propionate is activated to propionyl-CoA via a propionate kinase. In a newly enriched propionate oxidizer (MPOB) propionate was activated with a propionate: acetyl-CoA HS-CoA transferase (Houwen et al. 1990; Plugge et al. 1993). In all the syntrophic cultures investigated thus far, the decarboxylation of oxaloacetate is coupled to the carboxylation of propionyl-CoA via a transcarboxylation. During propionate oxidation, reducing equivalents are formed in the oxidations of succinate to fumarate, malate to oxaloacetate and pyruvate to acetyl-CoA; these reducing equivalents are formed at the level of $FADH_2$, NADH, and ferredoxin.

The mechanism of 'energy sharing' between the different bacteria in the coculture is quite intriguing. The $\Delta G^{\circ\prime}$ of the conversion of propionate to methane and carbondioxide is about − 56 kJ/mol propionate, the equivalent of about 1 ATP, which has to be shared by the three bacterial species:

$$\begin{aligned} &Propionate^- + 1.75H_2O \rightarrow \\ &1.75CH_4 + 1.25HCO_3^- + 0.25H^+ \\ &\Delta G^{\circ\prime} = -56.6kJ/mol\ propionate \end{aligned} \qquad (2)$$

Under conditions which are normally prevailing in anoxic bioreactors each of the bacterial species has about −20 kJ/mol available (Stams et al. 1989). Propionate-oxidizing bacteria form one ATP during the conversion of acetyl-CoA to acetate (Fig. 8). $FADH_2$ oxidation coupled to the reduction of protons or bicarbonate requires metabolic energy, likely via reversed electron transport. It can be speculated that 2/3 ATP is required for this, resulting in a net ATP formation of 1/3 ATP per propionate. The amount of ATP which *Wolinella succinogenes* gains from the reduction of fumarate with H_2 or formate is 2/3 ATP (Kröger et al. 1993). Recently, we have studied the effect of fumarate on the metabolism of the propionate oxidizer MPOB (Stams et al., 1993; Plugge et al. 1993). This bacterium was able to couple the degradation of propionate to the reduction of fumarate to succinate; 1 mol of propionate and 3 mol of fumarate yielded 1 mol of acetate and 3 mol of succinate. In addition, the bacterium could grow by reduction of fumarate with hydrogen or formate as electron donors. The molar growth yield of this bacterium was similar to that of *W. succinogenes*, indicating that this bacterium also yields 2/3 ATP from the reduction of fumarate (Van Kuijk, unpublished results). Therefore, it seems likely that 2/3 ATP is needed for the succinate oxidation step in the catabolism of propionate.

In methanogens, energy is conserved by the formation of proton- and sodium-gradients (Thauer 1990; Gottschalk & Blaut 1990). Four reduction steps are involved in methanogenesis from H_2 +

CO_2: CO_2 reduction to formyl-methanofuran ($\Delta G^{\circ\prime}$ = + 16 kJ/mol), methenyl-tetrahydromethanopterine (methenyl-H_4MPT) reduction to methylene-H_4MPT ($\Delta G^{\circ\prime}$ = − 5 kJ/mol), methylene-H_4MPT reduction to methyl-H_4MPT ($\Delta G^{\circ\prime}$ = − 20 kJ/mol) and methyl-Coenzyme M reduction to methane ($\Delta G^{\circ\prime}$ = −85 kJ/mol). The endergonic reduction of CO_2 requires metabolic energy, probably in the form of a sodium gradient (Kaesler & Schönheit, 1989), whereas energy is conserved in a sodium and proton gradient which are formed in the reduction of methylene-H_4MPT and subsequent transmethylation of methyl-H_4MPT, and in the reduction of methyl-CoM, respectively (Kaesler & Schönheit, 1989; Thauer 1990; Gottschalk & Blaut 1990). At a high hydrogen partial pressure, the reactions are sufficiently exergonic to allow the formation of a proton gradient and a net sodium gradient. However, at a low hydrogen partial pressure only the reduction of methyl-CoM is sufficiently exergonic to drive ATP synthesis. A net build up of a sodium gradient is not possible any more, and it is even likely to assume that ATP is required to create a sodium gradient to pull the highly endergonic reduction of CO_2 to formyl-methanofuran. The result would be the net synthesis of 1/3 ATP per CH_4 which is formed.

The amount of energy which can be conserved by aceticlastic methanogens does not depend on the concentration of hydrogen or formate. In these methanogens acetate is first activated to acetyl-CoA. Acetyl-CoA is cleaved into a methyl group and a carbonyl group, which subsequently are reduced to methane and oxidized to carbon dioxide, respectively. During this electron flow, energy conservation may take place by the formation of an ion gradient. It can be calculated that under normal physiological conditions aceticlastic methanogens can derive 1/3 ATP per acetate which is metabolized. However, the energy metabolism of aceticlastic methanogens is not fully understood, especially because *Methanosarcina* and *Methanothrix* have different ATP requirements for the activation of acetate to acetyl-CoA (Jetten et al. 1992).

The phylogenetic position of *S. wolinii* was analyzed by us (Harmsen et al. 1993). Surprisingly, this bacterium clustered with sulfate-reducing bacteria, with *Desulfomonile tiedjei* and *Desulfoarculus baarsii* as the most related species. Dörner (1992) showed that this bacterium indeed was able to couple propionate oxidation to sulfate reduction. The mesophilic bacterium MPOB was analyzed as well. This bacterium was closely related with *S. wolinii*, and it was also able to reduce sulfate. However, when cocultured in the presence of sulfate with *Desulfovibrio* G11, it preferred to grow syntrophically (Stams, unpublished results). Because of these findings it would be interesting to reinvestigate why other propionate-oxidizing sulfate reducers cannot grow syntrophically. *Desulfobulbus propionicus* and *D. elongatus* oxidize propionate to acetate with sulfate. In addition, they can grow with hydrogen plus sulfate (Widdel 1988; Widdel & Pfennig 1982; Samain et al. 1984), while in the absence of sulfate these bacteria are able to reduce acetate + CO_2 to propionate with hydrogen as electron donor (Laanbroek et al. 1982). Samain et al. (1986) have analyzed enzyme activities in *D. elongatus* and concluded that the hydrogenase activities might be too low to allow formation of hydrogen from propionate. Another explanation might be that the tested *Desulfobulbus* sp. cannot dispose of reducing equivalents as formate.

Syntrophic butyrate oxidation

As with propionate three different types of bacteria, a butyrate-degrading acetogen and two methanogens, are required for a complete mineralization of butyrate (Table 1). McInerney et al. (1981) enriched and characterized *Syntrophomonas wolfei*, a bacterium which degraded butyrate and some other short-chain fatty acids syntrophically. Several butyrate-oxidizing bacteria were described since, such as the spore-forming bacterium *Syntrophospora bryantii* (former *Clostridium bryantii*) (Stieb & Schink 1985; Zhao et al. 1989), strain SF-1 (Shelton & Tiedje 1984) and *Syntrophomonas sapovorans* (Roy et al. 1986). The latter bacterium can oxidize butyrate and saturated and unsaturated long-chain fatty acids. Thermophilic butyrate-degrading cocultures have been described as well (Henson & Smith 1985; Ahring & Westermann 1987a, 1987b, 1988).

In *S. wolfei* and *S. bryantii*, butyrate is converted to acetate by β-oxidation (Wofford et al. 1986; Schink 1992; Fig. 8). Butyrate is activated to butyryl-CoA, which then is converted to 2 acetyl-CoA, via two oxidation steps. One acetyl-CoA is used for the activation of butyrate, whereas the other yields one ATP in the conversion to acetate. In the oxidation of butyryl-CoA to crotonyl-CoA and 3-hydroxybutyryl-CoA to acetoacetyl-CoA reducing equivalents at the level of $FADH_2$ and NADH are formed, respectively. In a similar fashion as described above for propionate oxida-

tion, part of the ATP has to be used to pull the oxidation of butyryl-CoA. Dörner (1992) demonstrated that the butyryl-CoA dehydrogenase and the hydrogenase of *S. wolfei* were partly membrane-bound, and that hydrogen formation in BrES-inhibited cultures could be inhibited by the addition of protonophores, indicating that a reversed electron transfer mechanism could occur in this organism as well. The $\Delta G^{\circ\prime}$ of the complete mineralization of butyrate is about − 80 kJ/mol butyrate:

$$\begin{aligned} &Butyrate^- + 2.5H_2O \rightarrow 2.5CH_4 + \\ &1.5HCO_3^- + 0.5H^+ \\ &\Delta G^{\circ\prime} = -81.7kJ/mol\,butyrate \end{aligned} \quad (3)$$

Schink & Thauer (1988) calculated that under conditions which are realistic for an anoxic reactor, the $\Delta G'$ of each of the reactions is about − 20 kJ/mol, again indicating that each of the bacterial species has 1/3 ATP available for growth.

Syntrophic butyrate-degrading bacteria are able to couple butyrate oxidation to the reduction of unsaturated alkanes (Kasper et al. 1987). In addition, they are able to ferment crotonate and some other unsaturated short-chain fatty acids (Beaty & McInerney 1987; Amos & McInerney 1990; Zhao et al. 1990; Dörner 1992). *S. wolfei* ferments crotonate to acetate, butyrate and caproate, whereas *S. bryantii* only forms acetate and butyrate in the expected ratio of 2 to 1 (Dong, unpublished results). NADH formed in the oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA is used to reduce crotonyl-CoA to butyryl-CoA. Growth on crotonate was used to obtain *S. wolfei* and *S. bryantii* in pure culture (Beaty & McInerney 1987; Zhao et al. 1990, 1993).

The phylogenetic position of syntrophic butyrate-degrading bacteria was investigated. Because *Clostridium bryantii* was phylogenetically quite distinct from other clostridia it was renamed to *Syntrophospora bryantii* (Zhao et al. 1990). Recently, even a separate family, Syntrophomonaceae, was created to accommodate sporeforming and non-sporeforming syntrophic butyrate-degrading bacteria (Zhao et al. 1993).

Syntrophic acetate oxidation

Acetate can be degraded to methane and carbon dioxide by mesophilic and thermophilic *Methanosarcina* and *Methanothrix* species (Jones 1991; Whitman et al. 1992; Zinder et al. 1984; Huser et al. 1982;). However, Zinder & Koch (1984) observed that in a bioreactor operated at 60 °C acetate was not cleaved by methanogens but was degraded syntrophically. A mesophilic acetate-degrading coculture was described as well (Blomgren et al. 1990). In these cultures acetate is oxidized to 2 CO_2 and 4 H_2, the H_2 being used by methanogens for the reduction of carbon dioxide to methane.

Syntrophic acetate conversion has been reviewed recently by Zinder (1994). A thermophilic acetate-degrading bacterium was obtained in pure culture with pyruvate as substrate. The bacterium appeared to be a homoacetogen which grows on H_2 + CO_2, formate, CO and betaine (Lee & Zinder 1988a,1988b). The bacterium was nick-named 'Reversibacter' because of its ability to grow both by formation and by oxidation of acetate. Enzyme measurements and inhibition studies with cyanide showed that the acetyl-CoA cleavage pathway is involved in the formation and the degradation of acetate (Lee & Zinder 1988c). A similar pathway is also involved in sulfate-dependent oxidation of acetate by some sulfate-reducing bacteria (Schauder et al. 1986,1989; Spormann et al. 1988). In acetate oxidation, reducing equivalents are formed in 4 reactions: methyl-tetrahydrofolate (methyl-THF) oxidation to methylene-THF ($\Delta G^{\circ\prime}$ = + 40.1 kJ/mol), methylene-THF oxidation to methenyl-THF ($\Delta G^{\circ\prime}$ = + 23 kJ/mol), formate oxidation to CO_2 ($\Delta G^{\circ\prime}$ = + 1.3 kJ/mol) and CO oxidation to CO_2 ($\Delta G^{\circ\prime}$ = −16.2 kJ/mol). The oxidation of methyl-THF requires metabolic energy, likely in the form of a sodium gradient (Schink 1992).

Syntrophic degradation of aromatic compounds

Several benzoate-degrading cultures have been described (Ferry & Wolfe 1976; Mountfort & Bryant, 1982; Mountfort et al. 1984; Belaich et al. 1990; Kamagata et al. 1992). *Syntrophus buswellii* growing in coculture with a methanogen or with a *Desulfovibrio* sp. was characterized (Mountfort & Bryant 1982; Mountfort et al. 1984). A reductive pathway was proposed to be involved in the syntrophic degradation of benzoate. However, thus far only a few enzyme activities were measured in cell extracts of *S. buswellii* (Dörner 1992). The pathway is assumed to be similar to the pathway of other benzoate-degrading anaerobes (Evans & Fuchs 1988; Schink et al. 1992). The benzene ring is first reduced, and ring fission occurs as

a result of a kind of β-oxidation (Fig. 9). The dicarboxylic acid which is formed is then degraded further via β-oxidation to 3 acetate and HCO_3^-. The degradation proceeds via CoA derivatives. Recently, methanogenic enrichments were obtained with long-chain dicarboxylic acids as substrates (Matthies & Schink 1993). These compounds and fatty acids seem not to be utilized by *S. buswellii* (Mountfort & Bryant 1982). However, this bacterium is able to grow on crotonate in the absence of methanogens (Dörner 1992).

In mixed microbial systems degradation of phenol was demonstrated (Knoll & Winter 1987; Londry & Fedorak 1992). Syntrophic degradation of monohydroxybenzoates (Tschech & Schink 1986), catechol & hydroquinones (Szewzyk & Schink 1989) and aminobenzoate (Schnell & Schink 1992) were described as well. The Gibbs free energy changes of the degradation of hydroxylated aromatic compounds is largely dependent on the number of hydroxygroups (Holliger et al. 1988). The degradation of 2-hydroxybenzoate could already be achieved with the poor hydrogen-scavenging *Acetobacterium woodii* (Tschech & Schink 1986).

Xenobiotic aromatic compounds may also be degraded by syntrophic associations. The anaerobic degradation of 3-chlorobenzoate has been studied best. For detailed information the reader is referred to the review of Mohn & Tiedje (1992). Four bacterial species are involved in its degradation, a bacterium which converts 3-chlorobenzoate to benzoate, and three organisms which are required for benzoate degradation (Shelton & Tiedje 1984):

	$\Delta G^{\circ\prime}$
3 chlorobenzoate$^-$ + H_2 → benzoate$^-$ + H^+ + Cl^-	− 125 kJ/mol
benzoate$^-$ + 7 H_2O → 3 acetate$^-$ + HCO_3^- + 3H^+ + 3 H_2	+ 58.9 kJ/mol
2 H_2 + 0.5 HCO_3^- → 0.5 CH_4 + 1.5 H_2O	− 67.8 kJ/mol
3 acetate$^-$ + 3 H_2O → 3 CH_4 + 3 HCO_3^-	− 93 kJ/mol
3-chlorobenzoate$^-$ + 8.5 H_2O → 3.5 CH_4 + 3.5 HCO_3^- + Cl^- + 3.5 H^+	−226.9 kJ/mol

The dechlorinating organism, *Desulfomonile tiedjei* DCB1, is able to couple the reductive dechlorination of 3-chlorobenzoate to growth in a kind of dehalo-respiration. Interestingly, two organisms consume the reducing equivalents which are produced by the benzoate-degrading bacterium. The factors which determine the electron flow from the benzoate degrader to the methanogenic and the dechlorinating bacterium are not fully understood. Thermodynamically, the dechlorinating bacterium should have the highest affinity for hydrogen.

Syntrophic degradation of amino acids

In the last decade it has become clear that in methanogenic environments amino acids may be degraded by obligately and facultatively syntrophic associations. Nagase & Matsuo (1982) observed that in mixed methanogenic communities the degradation of alanine, valine and leucine was inhibited by an inhibition of methanogens, while Nanninga & Gottschal (1985) could stimulate the degradation of these amino acids by the addition of hydrogen-scavenging sulfate-reducers. The importance of interspecies electron transfer in the thermophilic degradation of protein was demonstrated as well (Örlygsson et al. 1993). *Clostridium sporogenes* (Wildenauer & Winter 1986; Winter et al. 1987, *Eubacterium acidaminophilum* (Zindel et al. 1988), *Acidaminobacter hydrogenoformans* (Stams & Hansen 1984), strain PA-1 (Barik et al. 1985), and *Selenomonas acidaminovorans* (Cheng et al. 1992) are examples of bacteria which can grow syntrophically on one or more of the amino acids aspartate, alanine, leucine, isoleucine, or valine.

The initial step in the degradation of alanine, valine, leucine and isoleucine is an NAD(P)-dependent deamination to the corresponding keto acid. The $\Delta G^{\circ\prime}$ of this reaction when coupled to hydrogen formation is about + 60 kJ/mol; methanogens are needed to pull this reaction. The keto acid is converted further by a probably ferredoxin-dependent oxidative decarboxylation to a fatty acid, a reaction which energetically is much more favorable ($\Delta G^{\circ\prime}$ is about −50 kJ/mol). Therefore, it is not surprising that the above mentioned organisms as far as they have been tested can grow in pure culture on keto acids. Keto acids were successfully used for the isolation of proton-reducing acetogens (Stams & Plugge 1990). Aspartate probably is not directly oxidized to oxaloacetate, but via fumarate and malate. In this conversion the malate oxidation step is most unfavorable; the $\Delta G^{\circ\prime}$ is + 47.7 kJ/mol.

Glutamate is fermented by *Acidaminobacter hydrogenoformans* to 2 acetate, 1 CO_2, NH_3 and 1 H_2, or to 1 propionate, 2 CO_2, NH_3 and 2 H_2:

	$\Delta G^{\circ\prime}$
Glutamate$^-$ + 3 H_2O → 2 acetate$^-$ + HCO_3^- + H^+ + NH_4^+ + H_2	−33.9 kJ/mol
Glutamate$^-$ + 4 H_2O → propionate$^-$ + 2 HCO_3^- + NH_4^+ + H^+ + 2 H_2	− 5.8 kJ/mol

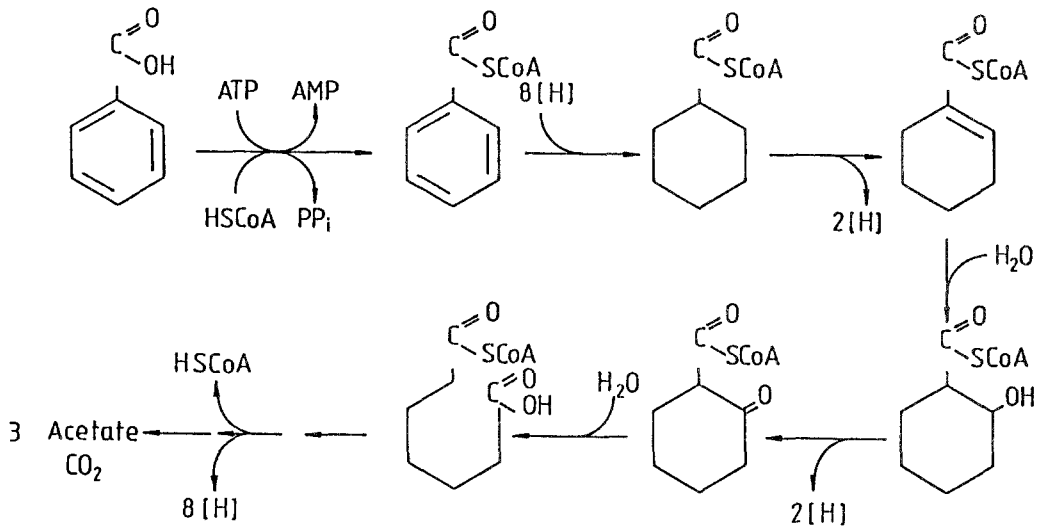

Fig. 9. Hypothetical pathway of syntrophic benzoate oxidation.

In pure culture, this bacterium is also able to form formate. However, in the presence of the hydrogen-utilizing *Methanobrevibacter arboriphilus*, formate is not formed. The ratio at which acetate and propionate are formed depends on the hydrogen partial pressure (Stams & Hansen 1984). Propionate formation is favored at a low hydrogen partial pressure, whereas at a high hydrogen partial pressure mainly acetate is formed. The energetic barrier in the metabolism is the reductive deamination of glutamate to α-ketoglutarate, ammonium and hydrogen ($\Delta G^{\circ\prime} = +59.9$ kJ/mol). The conversion of α-ketoglutarate to propionate, 2 CO_2 and H_2, which proceeds via succinyl-CoA, methylmalonyl-CoA and propionyl-CoA, is exergonic ($\Delta G^{\circ\prime} = -65.7$ kJ/mol). When the energetic barrier is taken away by cultivating the organism on α-ketoglutarate, propionate is also formed in pure culture.

We have studied how the pathways of acetate and propionate formation are regulated in this bacterium. Measurement of enzyme activities has indicated that the β-methylaspartate pathway as present in glutamate-fermenting *Clostridium* sp. (Buckel & Barker 1974; Barker 1981) is involved in the formation of acetate when the organism is grown in pure culture on glutamate (Stams & Hansen 1984). However, when the organism was grown on glutamate in the presence of *M. arboriphilus* or on α-ketoglutarate in pure culture, acetate seems to be formed via enzymes of a reversed citric acid cycle (Skrabanja unpublished results). The pathways of glutamate degradation in this organism are shown in Fig. 10.

We have isolated *Selenomonas acidaminovorans*, a moderately thermophilic organism which was able to ferment glutamate and a wide variety of other amino acids (Cheng et al. 1992). This organism formed the same products as *A. hydrogenoformans*. However, it produced propionate already in pure culture, and acetate most likely was formed only via enzymes of the reversed citric acid cycle. Recently, we have described a thermophilic propionate-oxidizing culture (Stams et al. 1992) which degraded glutamate syntrophically. This culture formed only one acetate per glutamate (Table 1). In this culture, glutamate is first oxidized to succinyl-CoA, which then is transferred directly to acetate, or indirectly via the intermediate formation of propionate (Fig. 8).

A. hydrogenoformans and *S. acidamovorans* are able to grow on histidine, ornithine, arginine, lysine and threonine when cocultured with hydrogenotrophic bacteria. Histidine was degraded to acetate and propionate. This amino acid can be degraded via the intermediate formation of glutamate (Gottschalk 1985). Remarkably, *S. acidaminovorans* was also able to grow in pure culture on arginine, and formed ornithine and citrulline as the only products. In this conversion carbamyl-phosphate is formed, which is further converted to yield ATP. Ornithine and citrulline could be degraded when the bacterium was cocultured with *M. thermoautotrophicum*.

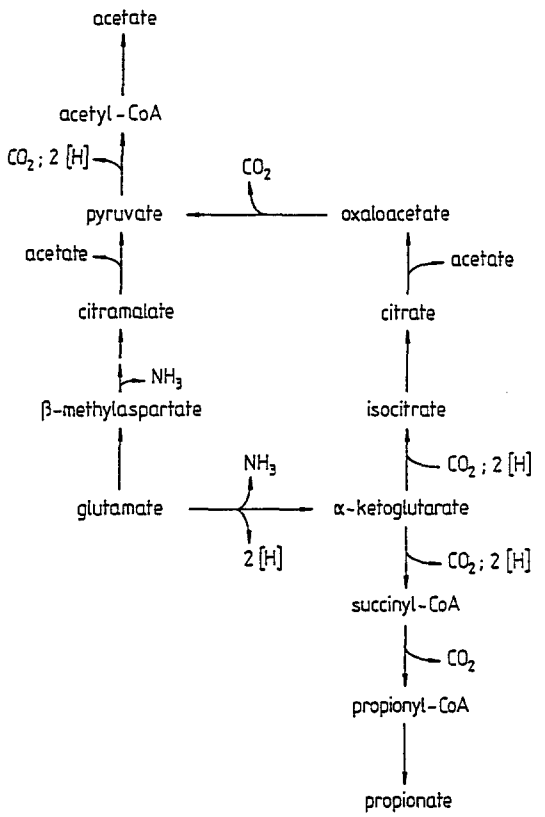

Fig. 10. Pathways involved in glutamate fermentation by *Acidaminobacter hydrogenoformans.*

Interspecies acetate transfer

Acetate consumption by methanogens may also be advantageous for anaerobic degradation processes. In cultures of *Methanothrix soehngenii* acetate threshold values as low as 10 μM have been measured (Jetten et al. 1990). Platen & Schink (1987) enriched an acetone-degrading bacterium in coculture with *Methanothrix.* Labelling studies have shown that acetone is degraded via a carboxylation to two acetate, the acetate being consumed by the methanogen:

$$\text{acetone} + HCO_3^- \rightarrow 2\ \text{acetate}^- + H^+ \quad \Delta G^{\circ\prime} = -30.1\ \text{kJ/mol}$$
$$2\ \text{acetate}^- + 2\ H_2O \rightarrow 2\ CH_4 + 2\ HCO_3^- \Delta G^{\circ\prime} = -31.0\ \text{kJ/mol}$$

Although the acetone carboxylation reaction is exergonic, low acetate concentrations appeared to be essential for the degradation of acetone. The biochemical mechanism of acetone degradation in this coculture is not yet known.

It can be calculated that e.g. propionate and butyrate oxidation cannot be pulled by low acetate concentrations alone. However, it has been shown that the addition of acetate inhibits propionate and butyrate oxidation, whereas the removal of acetate stimulated the growth of syntrophic propionate- and butyrate-oxidizing bacteria (Ahring & Westermann 1987b; Boone & Xun 1987; Stams et al. 1992; Van Lier et al. 1993). It can be calculated that if the acetate concentration is 10 μM rather than 10 mM the $\Delta G'$ of propionate and butyrate oxidation are lowered by 17.4 and 34.8 kJ/mol, respectively. In terms of fluxes of hydrogen and formate this would mean that the acetogenic bacteria can reach higher concentrations of formate and hydrogen before the $\Delta G'$ value become positive. Therefore, the removal of acetate may result in a steeper gradient of formate and hydrogen between the acetogens and the methanogens, resulting in increased fluxes.

Competitive interactions in methanogenic environments

Fermentative versus syntrophic degradation

Not all the compounds which can be degraded by syntrophic associations necessarily have to be degraded via such electron transfer-dependent processes. Compounds like ethanol, lactate and a number of amino acids can be fermented as well. Lactate, and ethanol (+ HCO_3^-) can be fermented by e.g. *Pelobacter propionicus* and *Desulfobulbus propionicus* to acetate and propionate (Schink 1984; Laanbroek et al. 1982, Stams et al. 1984, Samain et al. 1982). Lactate and ethanol (+CO_2) can also be fermented by homoacetogenic bacteria to solely acetate (Braun et al. 1981; Andreesen et al. 1970). In addition, *C. kluyveri* ferments ethanol (+ acetate) to butyrate (Bornstein & Barker 1948). Many amino acids, e.g. alanine, aspartate and glutamate can be fermented as well (Barker 1981; McInerney 1988). Generally, such fermenting bacteria have higher maximum specific growth rates than syntrophic associations. However, in e.g. methanogenic granular sludge the slow-growing acetogenic bacteria are often present in high numbers, indicating that these bacteria are well able to compete with fermenting bacteria at low substrate concentrations.

Competition between methanogens

A wide variety of different hydrogenotrophic and aceticlastic methanogens have been isolated from different methanogenic environments (Whitman et al. 1992). It is not always clear why a certain methanogen is the dominant species in a particular ecosystem. Some information is available concerning the competition for acetate between *Methanothrix* and *Methanosarcina* species. These two types of methanogens differ considerably in morphology and physiology (Jetten et al. 1992). *Methanosarcina* sp. show fastest growth at high acetate concentrations, and therefore they are easily enriched at high acetate concentrations. However, *Methanothrix* has a higher affinity for acetate, and they are most abundant in environments with a low acetate concentration, e.g. in methanogenic bioreactors. It was shown that the difference in affinity for acetate is caused by differences in the acetate-activating enzyme systems. *Methanosarcina* activates acetate to acetyl-CoA via an acetate kinase and a phosphotransacetylase:

	$\Delta G^{\circ\prime}$
Acetate + ATP → Acetyl-phosphate + ADP	+ 13.0 kJ/mol
Acetyl-phosphate + HS-CoA → Acetyl-CoA + P_i	− 9.0 kJ/mol
Acetate + ATP + HS-CoA → Acetyl-CoA + ADP + P_i	+ 4.0 kJ/mol

Methanothrix activates acetate with an acetyl-CoA synthetase; the pyrophosphate which is formed is cleaved into 2 phosphate and the AMP is converted to ADP by an adenylate kinase:

	$\Delta G^{\circ\prime}$
Acetate + ATP + HS-CoA → Acetyl-CoA + AMP + PP_i	− 5.9 kJ/mol
PP_i → 2 P_i	−21.9 kJ/mol
ATP + AMP → 2 ADP	0 kJ/mol
Acetate + 2 ATP + HS-CoA → Acetyl-CoA + 2 ADP + 2P_i	−27.8 kJ/mol

This acetate-activating reaction is more exergonic, and therefore lower acetate concentrations can be reached by *Methanothrix*.

The two types of aceticlastic methanogens have different pH optima, *Methanothrix* above 7 and *Methanosarcina* below 7. It was observed that *Methanothrix* was most numerous in propionate-adapted methanogenic sludge, while in ethanol-adapted sludge *Methanosarcina* was most abundant (Grotenhuis et al. 1991). This may have been due to the strong pH fluctuations in the UASB reactor degrading ethanol. If ethanol oxidation to acetate is not strictly coupled to the conversion of acetate by methanogens, the pH drops and the acetate concentration increases. These conditions favor growth of *Methanosarcina*. In the reactor degrading propionate such pH changes did not occur.

Future prospectives

The importance of syntrophic degradation is well established for compounds which cannot be degraded fermentatively, as is the case with e.g. propionate, butyrate and benzoate. However, the importance of syntrophic associations in degradation of other compounds is often underestimated. This particularly because bacteria are often isolated via enrichment techniques which select for fast growing fermenting bacteria. However, acetogenic bacteria growing in syntrophy with methanogens generally can conserve more energy than their fermenting counterparts growing on the same substrate. The role of syntrophic associations in the degradation of easily fermentable substrates can be elucidated if isolation techniques are applied which select for the most abundant organisms (direct dilution) or for bacteria with the highest affinity for substrates (energy limited chemostats).

Most acetogenic bacteria which grow syntrophically on fatty acids or aromatic compounds have been shown to grow on some other substrates in the absence of methanogens. This property allows to study the metabolism of these bacteria in detail. As acetogenic associations live at the border of what is thermodynamically possible their biochemical and bioenergetic mechanisms must be very efficient.

The role of formate and hydrogen transfer in syntrophic degradation still is a matter of controversy. Some acetogenic bacteria can grow syntrophically with methanogens which only use hydrogen, but some other acetogenic bacteria require methanogens which can use both hydrogen and formate. Inhibition by formate does not necessarily mean that formate transfer is the mechanism by which electrons are shuttled form the acetogen to the methanogen. Research with pure cultures of acetogenic bacteria can shed some light on the role of formate in interspecies electron transfer. In particular, enzymes involved in redox reactions, and the localization of electron transfer components have to be studied in more detail.

Highly chlorinated aliphatic and aromatic compounds are difficult to degrade aerobically, but these compounds are degraded anaerobically. Up to now most of the research on anaerobes was done with mixed microbial communities. Work on the anaerobic transformation of 3-chlorobenzoate has shown that such transformations require syntrophic cooperation as well. Interestingly, in these associations the dechlorinating bacteria participate in interspecies electron transfer. These interactions have to be studied in more detail on a biochemical level as well.

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