Energetics of syntrophic ethanol oxidation in defined chemostat cocultures

1. Energy requirement for H_2 production and H_2 oxidation

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Abstract. The ethanol-oxidizing, proton-reducing *Pelobacter acetylenicus* was grown in chemostat cocultures with either *Acetobacterium woodii, Methanobacterium bryantii*, or *Desulfovibrio desulfuricans*. Stable steady state conditions with tightly coupled growth were reached at various dilution rates between 0.02 and 0.14 h^{-1}. Both ethanol and H_2 steady state concentrations increased with growth rate and were lower in cocultures with the sulfate reducer < methanogen < homoacetogen. Due to the higher affinity for H_2, *D. desulfuricans* outcompeted *M. bryantii*, and this one *A. woodii* when inoculated in cocultures with *P. acetylenicus*. Cocultures with *A. woodii* had lower H_2 steady state concentrations when bicarbonate reduction was replaced by the energetically more favourable caffeate reduction. Similarly, cocultures with *D. desulfuricans* had lower H_2 concentrations with nitrate than with sulfate as electron acceptor. The Gibbs free energy (ΔG) available to the H_2-producing *P. acetylenicus* was independent of growth rate and the H_2-utilizing partner, whereas the ΔG available to the latter increased with growth rate and the energy yielding potential of the H_2 oxidation reaction. The "critical" Gibbs free energy (ΔG_c), i.e. the minimum energy required for H_2 production and H_2 oxidation, was -5.5 to -8.0 kJ mol^{-1} H_2 for *P. acetylenicus*, -5.1 to -6.3 kJ mol^{-1} H_2 for *A. woodii*, -7.5 to -9.1 kJ mol^{-1} H_2 for *M. bryantii*, and -10.3 to -12.3 kJ mol^{-1} H_2 for *D. desulfuricans*. Obviously, the potentially available energy was used more efficiently by homoacetogens > methanogens > sulfate reducers.

Key words: Homoacetogenesis – Methanogenesis – Sulfate reduction – Caffeate reduction – Nitrate reduction – Interspecies H_2 transfer – Affinity – H_2 threshold – "Critical" Gibbs free energy

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Methanogenic degradation of fatty acids, aromatics, and alcohols often depends on syntrophic cooperations in which different partner bacteria depend on each other and operate like one metabolic entity (Wolin 1974; Zehnder 1978; Bryant 1979; Dolfing 1988). The syntrophic dependence results from the unfavourable thermodynamics of, for example, ethanol fermentation to methanogenic substrates:

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2;$$
$$\Delta G^{\circ\prime} = +9.6 \text{ kJ mol}^{-1} \text{ ethanol}. \quad (1)$$

This reaction yields energy only if H_2 is maintained at a low concentration. Removal of H_2 can be accomplished by anaerobic H_2-oxidizing bacteria; the minimum H_2 concentration required depends on the respective energy-generating potential and decreases, e.g., from homoacetogens > methanogens > sulfate reducers (Cord-Ruwisch et al. 1988).

A theory has been developed which predicts tight coupling between the metabolism of the syntrophic partners through H_2 production and consumption kinetics (Archer and Powell 1985; Kreikenbohm and Bohl 1986), and suggests that the metabolic rate of the syntrophic coculture depends largely on the H_2-utilizing partner by controlling the energetically important H_2 concentration. So far, only two studies analyzed the energetic conditions of syntrophic partners using either ethanol (Seitz et al. 1988) or butyrate (Dwyer et al. 1988). Both studies suffer from applying static rather than chemostat culture techniques and, consequently, were unable to analyze the energetics prevailing during coupled syntrophic growth.

Therefore, we studied ethanol consumption in syntrophic chemostats of *Pelobacter acetylenicus* cocultured with different partner bacteria, and analyzed the energetics of the H_2-producing and H_2-consuming reactions.

Table 1. Partial reactions studied in chemostat cocultures and their Gibbs free energies under standard conditions

Reaction	$\Delta G^{\circ\prime}$ [kJ/mol H_2]
H_2 production	
(1) $CH_3CHOHCOO^- + 2\,H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 2\,H_2$	− 2.1
(2) $CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2\,H_2$	+ 4.8
H_2 oxidation	
(3) $4\,H_2 + 2\,HCO_3^- + H^+ \rightarrow CH_3COO^- + 4\,H_2O$	− 26.1
(4) $4\,H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 4\,H_2O$	− 33.9
(5) $4\,H_2 + SO_4^= + H^+ \rightarrow HS^- + 4\,H_2O$	− 38.0
(6) H_2 + caffeate → hydrocaffeate	− 85.5
(7) $4\,H_2 + NO_3^- + 2\,H^+ \rightarrow NH_4^+ + 3\,H_2O$	−149.9

Materials and methods

Organisms and cultivation

Methanobacterium bryantii strain MoH (DSM 863) and *Methanobrevibacter smithii* (DSM 861) were obtained from the German Collection of Microorganisms (DSM). *Pelobacter acetylenicus* strain WoAcy1 (DSM 2348), *Pelobacter acetylenicus* strain GhAcy1, *Pelobacter carbinolicus* strain GraBd1 (DSM 2380), *Acetobacterium woodii* strain NZva16 (Bache and Pfennig 1981), *Acetobacterium carbinolicum* (DSM 2925), *Methanospirilum hungatei* strain M1h, *Desulfovibrio desulfuricans* strain Essex 6 (DSM 642), and *Desulfovibrio vulgaris* strain Marburg (DSM 2119) were from the culture collection of our laboratory.

The bacteria were grown in bicarbonate-buffered mineral medium (Widdel and Pfennig 1981) with an initial pH of 7.2–7.3, 1 mM sodium acetate as carbon source, and 1.5 mM sodium sulfide as reducing agent. Electron donors and acceptors were added from sterile anaerobic stock solutions to a final concentration of 10 mM if not stated otherwise. Purity of cultures was routinely checked by phase contrast and fluorescence microscopy, and additionally by inoculation into complex AC medium (Difco) containing 4 mM pyruvate, 0.1% yeast extract, 5 mM sulfate and 5 mM nitrate.

Batch culture experiments (Cord-Ruwisch et al. 1988) were done at 28° C in shaken serum bottles (120 ml) containing 20 ml medium under an atmosphere of N_2:CO_2 (8:2) if grown with an organic electron donor, or H_2:CO_2 (8:2) if grown chemolithotrophically. For chemostat experiments, the pH-controlled system described by Cypionka (1986) was used, but without sulfide regulation. Cocultures were grown at 32° C under ethanol limitation, and steady state conditions were maintained for at least three volume changes of the cultures. The following strains were used for chemostat cultures: *P. acetylenicus* WoAcy1, *M. bryantii* MoH, *A. woodii* NZva16, *D. desulfuricans* Essex 6.

Analytical determinations

Gas samples were taken from the headspace of the culture vessels with gas-tight syringes, and analyzed for H_2, CH_4 and CO_2 as described by Seitz et al. (1988). H_2 partial pressures lower than 100 Pa were detected with a RGD2 detector (Techmation, Düsseldorf, FRG). Dissolved H_2 concentrations were calculated using the Bunsen solubility coefficients and molar gas volumes at the culture temperature. Bicarbonate concentrations were calculated from CO_2 and pH using published equations (Stumm and Morgan 1981). Ethanol, acetate, and lactate were determined by standard gas chromatographic procedures (Schink and Pfennig 1982). Caffeate and hydrocaffeate were determined by UV spectrophotometry at 190–350 nm wavelength (Hansen et al. 1988). Formate was analyzed by HPLC with a refractory index detector (Sykam, Gauting, FRG) using an Aminex HPX-87H column and 0.5 mM H_2SO_4 as eluent. Sulfate and nitrate were analyzed by ion chromatography (Sykam, Gauting, FRG) using a LCA AO1 column and 3 mM $NaHCO_3$/2 mM Na_2CO_3 as eluent.

The standard Gibbs free energies ($\Delta G^{\circ\prime}$) of the reactions studied are summarized in Table 1. They were calculated from the standard Gibbs free energies of formation (ΔG_f°) of the reactants and products (Thauer et al. 1977). The $\Delta G^{\circ\prime}$ of caffeate reduction to hydrocaffeate was from Grbic-Galic (1985). The Gibbs free energies (ΔG) under non-standard conditions which established in the cultures were calculated from the $\Delta G^{\circ\prime}$ of the individual reactions using the actually measured concentrations or partial pressures of the reactants and products, as well as the prevailing temperature and pH (Conrad et al. 1986).

Results

Rates, steady state concentrations, and *competition experiments*

Pelobacter acetylenicus was grown in ethanol-limited (9 mM in the reservoir) chemostat cultures using *Acetobacterium woodii*, *Methanobacterium bryantii*, or *Desulfovibrio desulfuricans* as H_2-utilizing partners. *M. bryantii* and also *A. woodii* (compare Buschhorn et al. 1989) were unable to use ethanol under the experimental conditions, and depended entirely on the H_2 produced by *P. acetylenicus* to grow. Stable steady state conditions for at least two volume changes were reached at dilution (growth) rates between 0.02 and 0.14 h^{-1}. Substrate and product concentrations, total cell mass and species composition remained stable at steady state. *D. desulfuricans* could grow with ethanol, but only at a low rate ($\mu <$ 0.022 h^{-1}; Szewzyk and Pfennig 1990). Stable coculture conditions were obtained at dilution rates between 0.025 and 0.09 h^{-1}. The μ_{max} was reached at 0.125 h^{-1} as determined by logarithmic washout.

In general, ethanol was stoichiometrically converted to acetate plus "homoacetate", methane or sulfide, with some of the acetate being assimilated into cell biomass. The recovery of carbon and electrons was between 81 and 108%. The rate of ethanol conversion to acetate and either sulfide, methane, or acetate by the syntrophic cocultures increased linearly with growth rate (Fig. 1). The ethanol conversion rate was independent of the type of syntrophic H_2-utilizing partner (Fig. 1).

Both ethanol and H_2 steady state concentrations increased with growth rate (Fig. 2). H_2 was formed as a

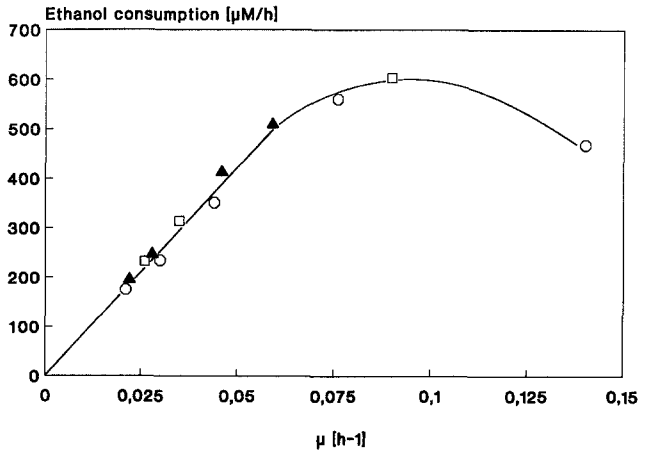

Fig. 1. Rates of ethanol consumption in ethanol-limited chemostat cocultures of *Pelobacter acetylenicus* plus either *Acetobacterium woodii* (○), *Methanobacterium bryantii* (▲), or *Desulfovibrio desulfuricans* (□)

trace intermediate. Ethanol and H_2 concentrations were nearly linearly correlated to each other. At dilution rates lower than 0.04 h^{-1}, both ethanol and H_2 concentrations were lower in cocultures with the sulfate reducer < methanogen < homoacetogen (Fig. 2).

Formate was also detected at low concentrations (30–250 μM) which did not change significantly with dilution rate or type of syntrophic partner. Formate could be oxidized by *A. woodii* and *D. desulfuricans*, but not by *M. bryantii*.

Inoculation of *M. bryantii* into a homoacetogenic chemostat coculture of *P. acetylenicus* with *A. woodii* resulted in a decrease of both H_2 and ethanol concentrations and in almost complete washout of *A. woodii* (Fig. 3). Inoculation of *M. bryantii* into a sulfate-reducing chemostat coculture with *D. desulfuricans*, on the other hand, did not result in changed H_2 or ethanol concentrations. *M. bryantii* finally was washed out of the culture fluid (not shown).

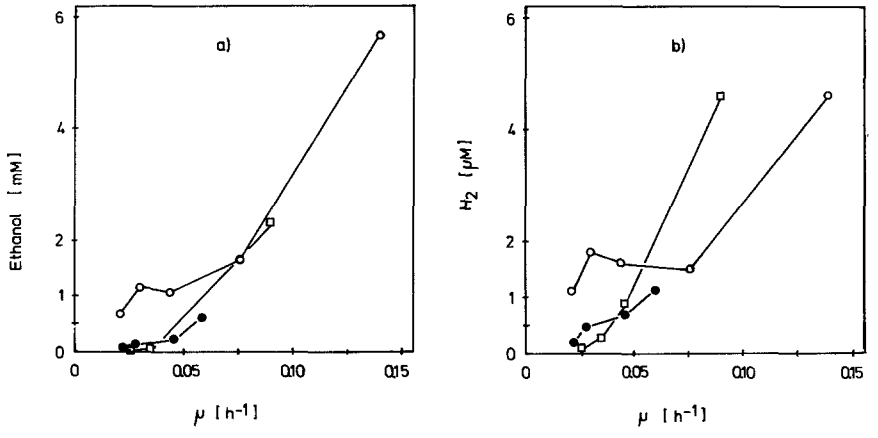

Fig. 2a, b. Steady-state concentrations of ethanol (**a**) and hydrogen (**b**) versus growth (dilution) rates (μ) in ethanol-limited chemostat cocultures of *P. acetylenicus* plus either *A. woodii* (○), *M. bryantii* (●), or *D. desulfuricans* (□)

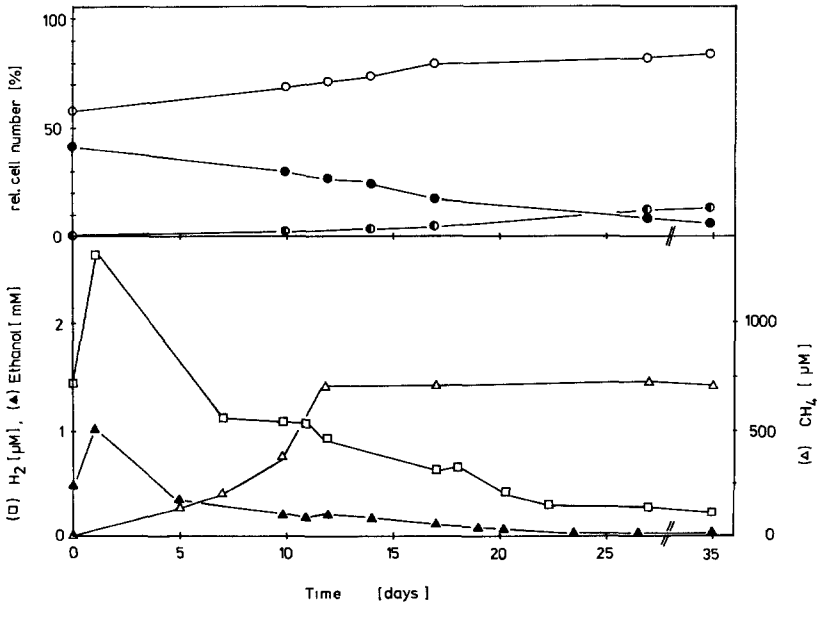

Fig. 3. Competition between *A. woodii* (●) and *M. bryantii* (◐) for H_2 during syntrophic oxidation of ethanol with *P. acetylenicus* (○) as H_2-producing partner organism. *M. bryantii* was inoculated at time zero into an ethanol-limited chemostat at μ = 0.022 h^{-1}

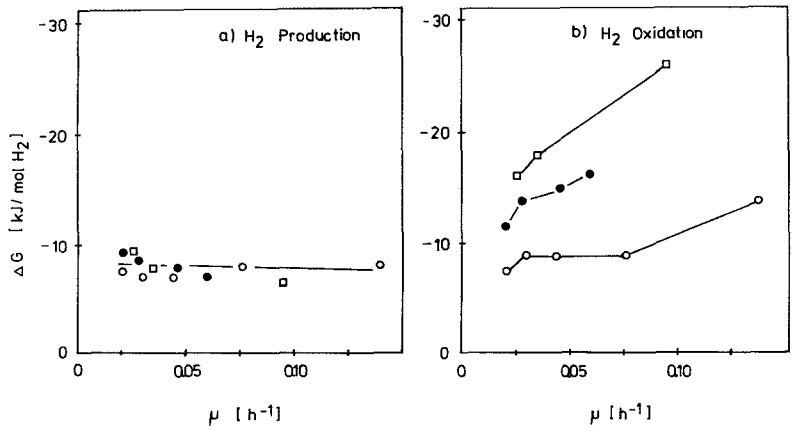

Fig. 4a, b. Gibbs free energies (ΔG) of H_2 production (**a**) and H_2 oxidation (**b**) during syntrophic ethanol oxidation determined in ethanol-limited chemostat cocultures of *P. acletylenicus* plus either *A. woodii* (○), *M. bryantii* (●), or *D. desulfuricans* (□) as partner organisms

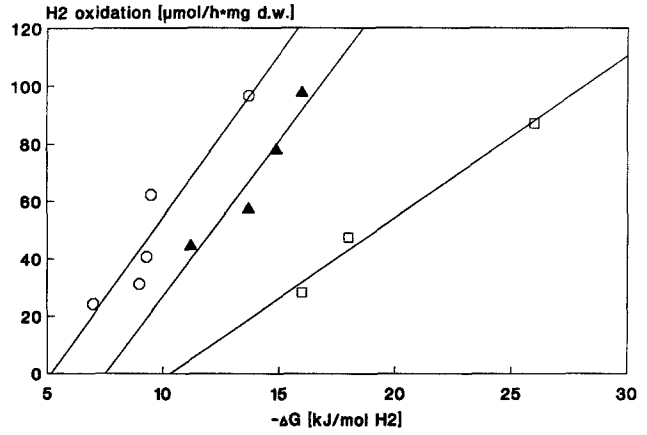

Fig. 5. Rates of H_2 oxidation versus Gibbs free energies of H_2 oxidation determined in ethanol-limited chemostat cocultures of *P. acetylenicus* plus either *A. woodii* (○), *M. bryantii* (▲), or *D. desulfuricans* (□) as partner organisms

Table 2. "Critical" Gibbs free energies (ΔG_c) extrapolated to zero H_2 oxidation and growth by different anaerobes growing in chemostat coculture with ethanol-fermenting *Pelobacter acetylenicus*

H_2 oxidizer	ΔG_c [kJ/mol H_2]	
	for oxidation[a]	for growth[b]
Acetobacterium woodii	− 5.1	− 6.3
Methanobacterium bryantii	− 7.5	− 9.1
Desulfovibrio desulfuricans	−10.3	−12.3

[a] Extrapolated to zero H_2 oxidation from Fig. 5
[b] Extrapolated to $\mu = 0$ from Fig. 4

In a homoacetogenic coculture with *A. woodii*, steady state H_2 concentrations decreased from 1.5 μM to 0.4 μM if caffeate was added. Caffeate reduction to hydrocaffeate quantitatively replaced the energetically less favourable reduction of bicarbonate to acetate. Stable steady state conditions were achieved at a dilution rate of 0.015 h^{-1} with 2.5 mM ethanol plus 6 mM caffeate, but not with 4.5 mM ethanol plus 10 mM caffeate.

Cocultures were also prepared with *D. desulfuricans* in the presence of nitrate as electron acceptor. Steady state H_2 concentrations were lower (0.02 μM) with nitrate than with sulfate (0.11 μM) at the same dilution rate (0.026 h^{-1}). However, stable syntrophic coupling could not be achieved since *P. acetylenicus* apparently was outcompeted by *D. desulfuricans* for ethanol and finally made up only 3−8% of the cells in the chemostat.

Energetics

The steady state Gibbs free energies (ΔG) available to each syntrophic partner under in situ conditions were calculated (Fig. 4). The ΔG available from ethanol conversion to acetate and H_2 was on average about −8 kJ mol^{-1} H_2. This value was independent of the growth rate and the type of syntrophic H_2-oxidizing partner (Fig. 4a). The ΔG values available from H_2 oxidation, however, increased with growth rate, and were higher for sulfate-reducers > methanogens > homoacetogens as H_2-oxidizing syntrophs (Fig. 4b), being in a range of −16 to −26 kJ mol^{-1} H_2, −11 to −16 kJ mol^{-1} H_2, and −7 to −14 kJ mol^{-1} H_2, respectively.

The specific rates of H_2 oxidation by the individual H_2 oxidizers were determined from the ethanol consumption rate at steady state (Fig. 1) and the cell titer of the H_2 oxidizers in the cocultures (Seitz et al. 1990). These specific H_2 oxidation rates increased with the Gibbs free energy available from H_2 oxidation under steady state conditions (Fig. 5), similar as the growth rate increased (Fig. 4). The "critical" Gibbs free energies (ΔG_c) extrapolated for zero H_2 oxidation and zero growth from Figs. 4 and 5, respectively, are summarized in Table 2.

"Critical" Gibbs free energies were also determined for the stationary phase of batch cultures of various pure cultures of H_2 producers and H_2 oxidizers (Table 3). Three different strains of ethanol-utilizing *Pelobacter* showed similar ΔG_c values of −5.2 to −5.5 kJ mol^{-1} H_2. Two strains of ethanol or lactate-utilizing *Desulfovibrio*, however, showed significantly more negative ΔG_c values of −13.9 to −18.2 kJ mol^{-1} H_2. As a trend, H_2-utilizing *A. woodii, M. bryantii* and *D. desulfuricans* showed more negative ΔG_c if an acceptor with a more positive redox potential was used. On the other hand, differences were

Table 3. "Critical" Gibbs free energies (ΔG_c) in batch cultures of various H_2-producing and H_2-oxidizing bacteria using different electron donors and acceptors

Strain	Reaction	Electron donor and acceptor	ΔG_c [kJ/mol H_2]
H_2-Producers			
D. desulfuricans	(1)	lactate + H^+	− 16.9
D. vulgaris	(1)	lactate + H^+	− 15.0
D. desulfuricans	(2)	ethanol + H^+	− 18.4
D. vulgaris	(2)	ethanol + H^+	− 13.9
P. acetylenicus	(2)	ethanol + H^+	− 5.5
P. acetylenicus GhAcyl	(2)	ethanol + H^+	− 5.2
P. carbinolicus	(2)	ethanol + H^+	− 5.3
H_2-Oxidizer			
A. woodii	(3)	$H_2 + HCO_3^-$	− 5.2
A. carbinolicum	(3)	$H_2 + HCO_3^-$	− 7.3
M. bryantii	(4)	$H_2 + HCO_3^-$	− 7.2
Methanospirillum hungatei	(4)	$H_2 + HCO_3^-$	− 7.2
Methanobrevibacter smithii	(4)	$H_2 + HCO_3^-$	− 11.1
D. desulfuricans	(5)	$H_2 + SO_4^{2-}$	− 11.0
A. woodii	(6)	H_2[a] + caffeate	− 15.6
D. desulfuricans	(7)	$H_2 + NO_3^-$	−117.4

[a] Data from a chemostat coculture with *P. acetylenicus* growing with 2.5 mM ethanol and 6 mM caffeate at $\mu = 0.015\ h^{-1}$

small among different species with the same type of H_2-oxidizing reaction.

Discussion

Tight syntrophic coupling was established between *Pelobacter acetylenicus* and either *Acetobacterium woodii*, *Methanobacterium bryantii*, or *Desulfovibrio desulfuricans* in ethanol-limited chemostat cultures. Ethanol was stoichiometrically oxidized to acetate with reduction of bicarbonate to either acetate or CH_4, or sulfate to sulfide, respectively. H_2 and formate were formed only in traces. Tightly coupled syntrophic chemostat cocultures were also reported for lactate or ethanol fermentation by *D. vulgaris* plus *Methanosarcina barkeri* (Traore et al. 1983; Tatton et al. 1989) and for butyrate fermentation by a thermophilic triculture (Ahring and Westermann 1987). Archer and Powell (1985) observed that growth curves of syntrophic batch cocultures were similar to those of monocultures, and that the growth rates of ethanol-fermenting cocultures (*D. vulgaris* plus various H_2-oxidizing methanogens) were related to those of the H_2-oxidizing syntrophic partner. Dwyer et al. (1988) observed in butyrate-fermenting cocultures (strain NSF-2 plus *Methanospirillum hungatei* or *Desulfovibrio* sp.) that H_2 concentrations decreased concomitant with butyrate concentrations; however, this occurred only during the stationary phase indicating that in batch cocultures tight syntrophic coupling may be achieved not until the end of exponential growth. The results of our chemostat experiments show that growth rates of the syntrophic partners were equal in steady state, and that H_2 and ethanol concentrations were linearly related to each other. Our results thus confirm the models that predict tight coupling of growth rates and rate-limiting substrate concentrations for syntrophic partners (Powell 1984, 1985; Kreikenbohm and Bohl 1986).

At steady state and low growth rates, both H_2 and ethanol concentrations were higher in homoacetogenic > methanogenic > sulfate-reducing cocultures. H_2 concentrations further decreased in cocultures with caffeate-reducing *A. woodii*, and were still lower in cocultures with nitrate-reducing *D. desulfuricans*. It has been shown that the threshold of H_2-oxidizing anaerobic bacteria for H_2 decreases with increasing redox potential of the terminal electron acceptor (Cord-Ruwisch et al. 1988). A similar trend has been shown for the K_m (review by Conrad 1989) and may also be anticipated for the affinity (V_{max}/K_m) to H_2. Our results confirm this trend for steady state conditions in the chemostat. The steady state H_2 concentrations were about 10-fold higher than the H_2 threshold concentrations measured in batch cultures (Cord-Ruwisch et al. 1988) indicating that in the chemostat affinity is more important than threshold. Our results furthermore show that high affinity for H_2 obviously resulted also in high affinity for ethanol and vice versa. Hence, the ethanol conversion rate of the H_2-producing fermenter is apparently impaired by negative feedback of its product, similar as observed for syntrophic butyrate fermentation in batch culture (Dwyer et al. 1988).

Although ethanol concentrations in the various syntrophic cocultures were significantly different, the differences were too small to significantly affect ethanol conversion rates; rather, the latter increased proportionally with growth rate. Competition experiments, however, clearly demonstrated that sulfate reducers > methanogens > homoacetogens outcompeted each other as syntrophic partners. These results confirm that the small differences in steady state H_2 concentrations were

sufficient for successful competition of the H_2 oxidizer with the higher affintiy for H_2. The competition was most probably based on interspecies H_2 transfer rather than interspecies formate transfer (Thiele and Zeikus 1988), since *M. bryantii* was unable to utilize formate, but nevertheless outcompeted the potentially formate-utilizing *A. woodii*.

The thermodynamic analysis of our chemostat experiments allowed to characterize the energetic situation of each syntrophic partner. The H_2-producing bacterium experienced similar Gibbs free energies for ethanol fermentation, independent of the different growth rates and combinations with H_2-oxidizing syntrophic partners. The H_2 producer apparently was under energetic homeostasis. Energetic homeostasis has also been reported for the H_2 producer in butyrate-fermenting batch cocultures (Dwyer et al. 1988). The energy demand ($\Delta G = -8$ kJ/mol H_2) for steady-state H_2 production in coculture was only slightly higher than the minimum energy required ($\Delta G_c = -5.3$ kJ/mol H_2) for ethanol fermentation in static monoculture of *P. acetylenicus*. However, the energy demand was significantly higher in either ethanol- or lactate-fermenting monocultures of *D. desulfuricans* or *D. vulgaris*, indicating that the level of energy homeostasis in syntrophic H_2 producers may be species-specific.

The H_2-oxidizing syntrophic partners were not under energetic homeostasis. Instead, the steady state Gibbs free energies ($-\Delta G$) increased with increasing growth rates and with specific rates of H_2 oxidation indicating that the energy demand of the H_2 oxidizers became higher with increasing H_2 turnover. Furthermore, the energy demand of the syntrophic H_2 oxidizer increased with increasing redox potential of the final electron acceptor. This observation was confirmed by the H_2 threshold concentrations and "critical" Gibbs free energies determined in monocultures of various anaerobic H_2 oxidizers. The minimum energy ($-\Delta G_c$) required for H_2 oxidation decreased from nitrate reduction > caffeate reduction > sulfate reduction > methanogenesis > homoacetogenesis and thus, show the same trend as that of the standard Gibbs free energies ($-\Delta G^{\circ\prime}$) of the individual reactions. Obviously, the potentially available energy was used more efficiently by homoacetogenesis > methanogenesis > sulfate-reduction. This trend was reaction-specific rather than bacterium-specific, since different strains of homoacetogens or methanogens exhibited the same energy demand; on the other hand, nitrate reduction and caffeate reduction exhibited higher energy demands than sulfate reduction or homoacetogenesis by the same bacterium. The dependence of the energy demand for H_2 oxidation on the terminal electron acceptor and on the growth rate indicates that energy generation can be uncoupled from H_2 turnover in the H_2-oxidizing syntrophs. This conclusion is consistent with the cellular growth yields observed during our experiments (Seitz et al. 1990) and in H_2-oxidizing methanogens (Schönheit et al. 1980; Fardeau and Belaich 1986).

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