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Energetics of Syntrophic Propionate Oxidation in Defined Batch and Chemostat Cocultures

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Propionate consumption was studied in syntrophic batch and chemostat cocultures of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*. The Gibbs free energy available for the H_2-consuming methanogens was < -20 kJ mol of CH_4^{-1} and thus allowed the synthesis of 1/3 mol of ATP per reaction. The Gibbs free energy available for the propionate oxidizer, on the other hand, was usually > -10 kJ mol of propionate^{-1}. Nevertheless, the syntrophic coculture grew in the chemostat at steady-state rates of 0.04 to 0.07 day^{-1} and produced maximum biomass yields of 2.6 g mol of propionate^{-1} and 7.6 g mol of CH_4^{-1} for *S. fumaroxidans* and *M. hungatei*, respectively. The energy efficiency for syntrophic growth of *S. fumaroxidans*, i.e., the biomass produced per unit of available Gibbs free energy was comparable to a theoretical growth yield of 5 to 12 g mol of ATP^{-1}. However, a lower growth efficiency was observed when sulfate served as an additional electron acceptor, suggesting inefficient energy conservation in the presence of sulfate. The maintenance Gibbs free energy determined from the maintenance coefficient of syntrophically grown *S. fumaroxidans* was surprisingly low (0.14 kJ h^{-1} mol of biomass C^{-1}) compared to the theoretical value. On the other hand, the Gibbs free-energy dissipation per mole of biomass C produced was much higher than expected. We conclude that the small Gibbs free energy available in many methanogenic environments is sufficient for syntrophic propionate oxidizers to survive on a Gibbs free energy that is much lower than that theoretically predicted.

Propionate is an important intermediate in the conversion of organic matter to methane and carbon dioxide. In methanogenic environments, the degradation of propionate to acetate and CO_2 may account for 6 to 35 mol% of the total methanogenesis (17). Propionate oxidation itself is energetically very unfavorable under standard thermodynamic conditions (see Table 1). Under methanogenic conditions, proton-reducing acetogenic bacteria are only able to gain energy from this reaction when the concentration of products is kept low. Thus, the degradation of propionate is only accomplished in obligate syntrophic consortia of proton-reducing acetogenic bacteria and methanogenic archaea (27, 28, 33). So far, three syntrophic propionate-oxidizing bacteria and some highly purified enrichment cultures have been described (3, 11, 20, 21, 22, 32, 43, 44, 49).

Studies have shown that most of the known syntrophic propionate-oxidizing bacteria degrade propionate via the methylmalonyl-coenzyme A (CoA) pathway (14, 25, 43). During the oxidation of propionate in the methylmalonyl-CoA pathway, electrons are released in three reactions, namely, the oxidation of succinate to fumarate, malate to oxaloacetate, and pyruvate to acetyl-CoA (25). In methanogenic environments, the H_2 partial pressure is low enough to allow the direct reduction of protons with the electrons released during the oxidation of pyruvate and malate. However, the H_2 partial pressure is not sufficient to allow this reduction during the oxidation of succinate to fumarate. It was hypothesized that the electrons released during the oxidation of succinate are shifted to a lower redox potential via reversed electron transport. This transport would be driven by the hydrolysis of 2/3 mol of ATP (27, 28, 37). Some evidence has been obtained for the presence of a reversed electron transport system in syntrophic propionate-degrading bacteria (41). However, the methylmalonyl-CoA pathway yields only 1 mol of ATP via substrate level phosphorylation. Therefore, if such a reversed electron transport is occurring, only 1/3 mol of ATP per mol of propionate is left for growth. Under physiological conditions, the Gibbs free-energy change needed for ATP synthesis must amount to a minimum of 70 kJ mol of ATP^{-1}. Thus, the minimum Gibbs free-energy quantum that can generate 1/3 mol of ATP would amount to approximately -23 kJ mol of propionate^{-1}. It has been suggested that this amount of Gibbs free-energy change corresponds to the minimum energy quantum required to sustain microbial life (27, 28).

In several methanogenic environments, the apparent Gibbs free-energy change for propionate oxidation was on the order of -3 to -15 kJ mol of propionate^{-1} and thus was rather small (5, 19, 26, 46). This amount of free-energy change is less than the minimum energy quantum needed to sustain microbial life. It is not clear why such small free-energy changes are observed during the degradation of propionate. Most syntrophic propionate-oxidizing bacteria are not obligate syntrophs but are also able to grow on other substrates, such as fumarate, malate, and pyruvate, in the absence of a partner microorganism. A remarkable feature of the propionate-oxidizing *Syntrophobacter* species is their ability to couple the oxidation of propionate not only to an H_2-consuming syntrophic partner but also to the reduction of sulfate. In fact, phylogenetic analysis of *Syntrophobacter fumaroxidans* has revealed that this bacterium is indeed related to sulfate-reducing bacteria (12). Perhaps the ability to reduce sulfate is of importance to explain the energetics of syntrophic propionate-oxidizing bacteria.

Besides propionate, many alcohols, fatty acids, amino acids, and aromatic compounds are anaerobically degraded by syntrophy. In each case, the available free energy is relatively low and has to be shared between the two syntrophic partners (27, 28). The energetics of syntrophic interspecies H_2 transfer has

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been studied in defined cocultures of benzoate-, lactate-, ethanol-, propionate-, and butyrate-oxidizing fermenting bacteria with H_2-consuming methanogens (1, 8, 9, 30, 31, 28, 39, 45). Propionate oxidation, however, has not yet been investigated in continuous-culture experiments.

Therefore, we studied the energetics of propionate consumption in syntrophic cocultures of *S. fumaroxidans* and *Methanospirillum hungatei* in the absence and presence of sulfate by determining the Gibbs free energy available for both the propionate oxidizers and the H_2-consuming methanogens under steady-state conditions in batch and chemostat cultures. The growth yields and maintenance coefficients of the syntrophic propionate oxidizer were also determined.

MATERIALS AND METHODS

Organisms and cultivation. *M. hungatei* JF1^T (DSM 864) and *S. fumaroxidans* MPOB (DSM 10017) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

The microorganisms were grown in bicarbonate-buffered, sulfide-reduced mineral medium as described previously by Huser et al. (15). To 1 liter of medium, 1 ml of a vitamin solution (48) and 1 ml each of an acid and an alkaline trace elements solution were added (32). The vitamin solution was filter sterilized separately. The gas phase above the medium was N_2-CO_2 (80% to 20%) at 172 kPa, and the pH of the medium was 6.8 to 6.9. Substrates and other supplements were added from sterile anaerobic stock solutions. Pure cultures of *S. fumaroxidans* and *M. hungatei* were maintained on fumarate (40 mM) and hydrogen, respectively. Hydrogen was added to the headspace at an overpressure of 60 kPa. In the case of *M. hungatei*, 2 mM (each) formate, acetate, and isobutyrate were added as supplementary carbon sources. Culture purity was routinely checked by phase-contrast and fluorescence microscopy.

Batch and chemostat experiments. In batch experiments, pure cultures of *S. fumaroxidans* or defined cocultures of *S. fumaroxidans* and *M. hungatei* were cultivated in 1-liter serum bottles containing 500 ml of mineral medium with 40 mM propionate with or without sulfate (40 mM) under a gas phase of N_2-CO_2 (80% to 20%) at 172 kPa.

Continuous cultivation was performed in a 1-liter chemostat system as described by Cypionka (7) with a working volume of 800 ml (flushed headspace) or 980 ml (nonflushed headspace). The chemostat experiments were only performed with cocultures of *S. fumaroxidans* and *M. hungatei*. The cocultures were grown under propionate limitation, and steady-state conditions were maintained for at least three culture volume changes. Substrate and product conditions were monitored, and total cell mass and species composition were checked under steady-state conditions.

For inoculation of the batch and chemostat experiments, *S. fumaroxidans* and *M. hungatei* were pregrown on fumarate and hydrogen, respectively. Batch experiments with pure cultures of *S. fumaroxidans* were inoculated with 10% (vol/vol) *S. fumaroxidans*. Cocultures for batch and chemostat experiments were constructed by inoculating 10% (vol/vol) *S. fumaroxidans* and 10% (vol/vol) *M. hungatei*. All experiments were performed at 37°C.

Determination of specific cell mass. Batch experiments were done as described above. Pure cultures of *S. fumaroxidans* and *M. hungatei* were grown with propionate plus SO_4^{2-} and H_2, respectively. Growth was measured by monitoring the total cell protein (see below). Cells were counted by phase-contrast microscopy using a Helber counting chamber. Bacterial dry mass was determined gravimetrically (see below). Cell suspensions contained (per liter) 27.4 ± 1.8 and 27.5 ± 0.6 mg (dry weight [dw]) of cells of *S. fumaroxidans* and *M. hungatei*, respectively. The corresponding cell numbers were (10^8 per ml) 2.25 ± 0.81 and 2.33 ± 0.01. The corresponding specific cell masses (x_i; picograms [dw] per cell) 0.116 ± 0.037 and 0.119 ± 0.003.

Determination of growth parameters. The maximum specific growth rate (μ_{max}; per day) was calculated from the exponential part of the propionate depletion curves of batch cultures. The total growth yield of the pure and mixed cultures growing on propionate or on propionate plus sulfate in batch culture experiments was determined from the microbial cell dw (see below). The total growth yield in continuous culture was determined from the protein concentration at steady state by multiplication by a conversion factor (see below). The total biomass yield was calculated from the number of grams of dry biomass produced per mole of propionate degraded. For cocultures, the determined yield is the sum of the yields of both species participating in the degradation of propionate. Consequently, the measured yield is referred to as $Y_{Xtot\text{-}S}$ (grams of dw per mole of a substrate).

The dry mass of the propionate-degrading and methanogenic microorganisms (X_i; grams [dw] per liter) were calculated from the total cell mass (X_{tot}), the relative cell numbers (N_i; cells per milliliter) in the culture, and the specific cell masses (x_i; grams [dw] per cell) according to the following equations (26):

$$X_{MPOB} = X_{tot} \times x_{MPOB} \times N_{MPOB}/(x_{MPOB} \times N_{MPOB} + x_{Mhun} \times N_{Mhun}) \quad (1)$$

$$X_{tot} = X_{MPOB} + X_{Mhun} \quad (2)$$

$$1 = N_{MPOB} + N_{Mhun} \quad (3)$$

The growth yields of the propionate-degrading microorganisms ($Y_{MPOB\text{-}S}$) and hydrogenotrophic methanogens ($Y_{Mhun\text{-}P}$) in the batch incubations were calculated by relating the increase in the biomass concentration to the amount of propionate (S) degraded or methane (P) formed.

In the chemostat experiments, the maximum growth yield (Y_{MPOB}^{max}) and the maintenance coefficient (m_s; millimoles of propionate per hour per gram [dw]) were obtained from the regression parameters of the following relationship (24):

$$1/Y = 1/Y^{max} + m_s \times \mu \quad (4)$$

with Y the apparent growth yield at different dilution rates ($\mu = D$) in a chemostat.

Gibbs free-energy changes. Standard Gibbs free-energy changes (ΔG^0) for the individual steps in the degradation of propionate (see Table 1) were calculated from the standard Gibbs free energies of formation (ΔG_f^0) of the reactants and products (36), corrected for a temperature of 37°C by the Gibbs-Helmholtz equation. The Gibbs free energies (ΔG) in the cultures were calculated from the ΔG^0 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 (6.9).

Maintenance energy. The maintenance energy (m_E) was determined from measured values as described by Heijnen and VanDijken (13) and Tijhuis et al. (38). The measured maintenance coefficients (m_s), in millimoles of substrate (electron donor) per gram (dw) of biomass per hour, were converted to m_D, in moles of substrate C per mole of biomass C per hour. The available standard Gibbs free energy (ΔG_{av}^{01}; kilojoules per mole of substrate) was calculated from $\Delta G^{0'}$ (kilojoules per mole of substrate) of the reaction divided by the number of C atoms (three for propionate) and the degree of reduction ($\gamma_D = 4.67$ for propionate) of the substrate. The maintenance energy, in kilojoules per mole of biomass C per hour, was then calculated by the following equation:

$$m_E = m_D \gamma_D \times \Delta G_{av}^{01} \quad (5)$$

Tijhuis et al. (38) found that m_E can also be determined theoretically by the following equation:

$$m_E = 3.3 \times \exp[-6.94 \times 10^4/R(1/T - 1/298)] \quad (6)$$

Gibbs free-energy dissipation per mole of biomass C. The Gibbs free-energy dissipation per mole of biomass C (D_s^{01}/r_{Ax}; kilojoules per mole of biomass C) was calculated as described by Heijnen and VanDijken (13) by solving the macrochemical equation, which, for the syntrophic oxidation of propionate by a coculture of *S. fumaroxidans* and *M. hungatei*, can be written as follows:

$$f\,C_3H_5O_2^- + a\,NH_4^+ + b\,H_2O + 1CH_{1.8}O_{0.5}N_{0.2} + c\,C_2H_3O_2^- + d\,CH_4 + e\,CO_2 = 0 \quad (7)$$

The macrochemical equation for the oxidation of propionate by *S. fumaroxidans* can be written as follows:

$$f\,C_3H_5O_2^- + a\,NH_4^+ + b\,H_2O + 1CH_{1.8}O_{0.5}N_{0.2} + c\,C_2H_3O_2^- + d\,H_2 + e\,CO_2 = 0 \quad (8)$$

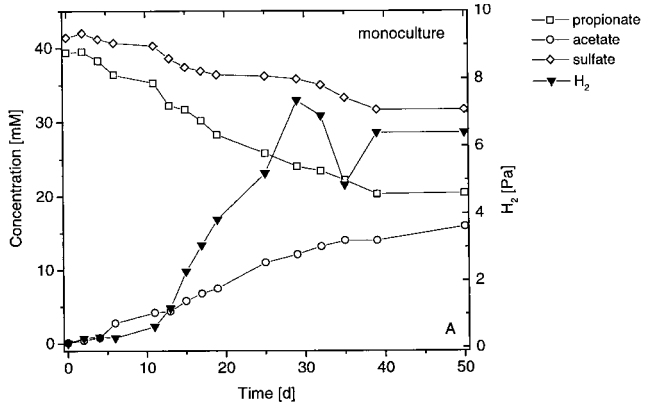
The coefficient f was calculated from the determined maximum growth yield (Y^{max}). The other five stoichiometric coefficients (a, b, c, d, e) were calculated from the five (C, H, O, N, and electric charge) conservation equations. D_s^{01}/r_{Ax} was then calculated by using the tabulated ΔG_f^0 for the reactants and products and $\Delta G_f^0 = -67$ kJ mol of biomass C^{-1} ($CH_{1.8}O_{0.5}N_{0.2}$) (13).

Heijnen and VanDijken (13) showed that D_s^{01}/r_{Ax} can also be theoretically predicted from the number of C atoms (C) and the degree of reduction (y_D) of the substrate C source as follows:

$$D_{sv}^{01}/r_{Ax} = 200 + 18(6 - C)^{1.8} + \exp\{[(3.8 - y_D)^2]^{0.16}(3.6 + 0.4C)\} \quad (9)$$

For propionate, the theoretical D_s^{01}/r_{Ax} is 426.6 kJ mol of biomass C^{-1}. This value can then be used to calculate a theoretical growth yield for the coculture of *S. fumaroxidans* plus *M. hungatei* and for *S. fumaroxidans* alone using the macrochemical equations and solving for the six stoichiometric coefficients by using the six (C, H, O, N, electric charge, and energy) conservation equations (13).

Analytical procedures. During microbial growth, samples were taken for analysis of substrate and product concentrations. H_2 and CH_4 were quantified by gas chromatography (6, 29). Propionate and acetate were measured by high-pressure liquid chromatography (18). Sulfate was analyzed by ion chromatography (2). Bacterial growth was monitored by protein determination. Cell pellets of 4-ml culture samples were resuspended in 1 ml of 1 M NaOH. After heating at 100°C for 15 min, the samples were treated further by the method of Bradford (4). Bovine serum albumin was used as the standard. The dw of a known culture volume was determined gravimetrically. The samples were gassed with N_2-CO_2



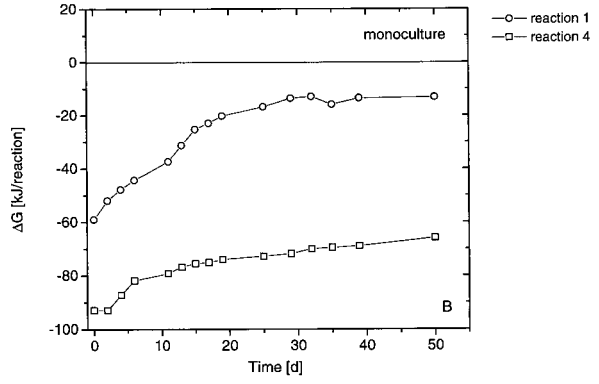
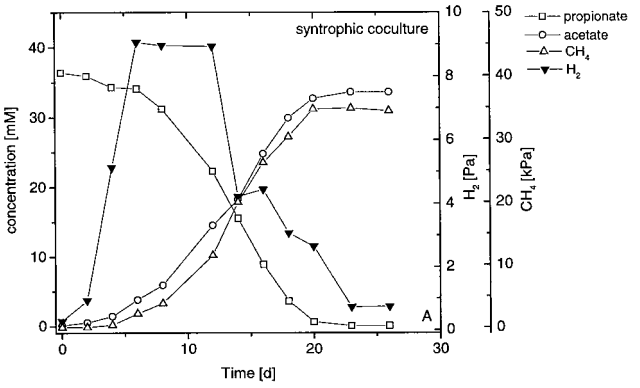


FIG. 1. Batch monoculture of *S. fumaroxidans* growing on propionate plus sulfate. (A) Changes in propionate (□), acetate (○), hydrogen (▼), and sulfate (◇). (B) Actual Gibbs free-energy changes of fermentative propionate oxidation (○) and sulfidogenic propionate oxidation (□).



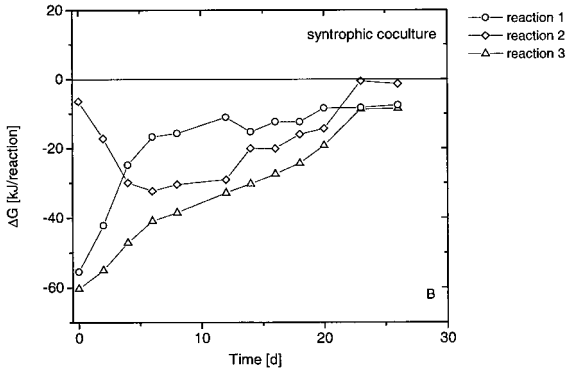


FIG. 2. Batch syntrophic coculture of *S. fumaroxidans* and *M. hungatei* growing on propionate. (A) Changes in propionate (□), acetate (○), hydrogen (▼), and methane (△). (B) Actual Gibbs free-energy changes of fermentative propionate oxidation (○), hydrogenotrophic methanogenesis (◇), and syntrophic propionate oxidation (△).

to remove H_2S, centrifuged, and washed twice with 50 mM ammonium acetate buffer (pH 6.0). The washed suspension was transferred into glass bottles and dried at 100°C to a constant weight. Cell numbers were counted by phase-contrast microscopy (see above). Conversion factors between protein content and biomass dw were determined by dividing the biomass dw by the protein content.

RESULTS

Batch experiments. The degradation of propionate by a pure culture of *S. fumaroxidans* in the presence of sulfate is shown in Fig. 1A. Propionate was stoichiometrically converted (Table 1) to acetate while sulfate was reduced. However, only part of the propionate was degraded. H_2 accumulated to about 7 Pa and remained at this level. A syntrophic culture of *S. fumaroxidans* and *M. hungatei* (syntrophic coculture) degraded propionate completely to acetate and CH_4 (Fig. 2A). During the incubation, H_2 transiently accumulated to about 9 Pa but decreased at the end to 0.8 Pa. A coculture grown on propionate plus sulfate (sulfidogenic coculture) degraded propionate almost completely to acetate (Fig. 3A). During the incubation, H_2 accumulated to about 4 Pa but again decreased at the end to 1.5 Pa.

The actual Gibbs free energies of the degradation of propionate by a pure culture of *S. fumaroxidans* in the presence of sulfate are depicted in Fig. 1B for both fermentative and sulfidogenic propionate degradation. The Gibbs free-energy val-

TABLE 1. Reactions involved in the degradation of propionate and their standard Gibbs free-energy changes, corrected for a pH of 6.9 and a temperature of 37°C[a]

Reaction	Equation[b]	$\Delta G^{0'}$ (kJ/reaction)
1. Propionate oxidation	$C_3H_5O_2$ (aq) + $2H_2O$ (l) → $C_2H_3O_2$ (aq) + CO_2 (g) + $3H_2$ (g)	+68.4
2. Hydrogenotrophic methanogenesis	$4H_2$ (g) + CO_2 (g) → CH_4 (g) + $2H_2O$ (l)	−125.9
3. Syntrophic oxidation of propionate	$C_3H_5O_2$ (aq) + $1/2H_2O$ (l) → $C_2H_3O_2$ (aq) + $3/4(CH_4$ (g) + $1/4CO_2$ (g)	−26.0
4. Sulfidogenic oxidation of propionate	$C_3H_5O_2$ (aq) + $3/4SO_4^{2-}$ (aq) + $3/4H^+$ → $C_2H_3O_2$ (aq) + CO_2 (g) + $3/4HS^-$ (aq) + H_2O (l)	−42.8

[a] Temperature correction was made with the Gibbs-Helmholtz equation. The applied ΔH_f^0 for propionate was −460.2 kJ mol^{-1}, which was calculated as described by Hanselmann (10).

[b] Abbreviations: aq, aqueous; l, liquid; g, gaseous.

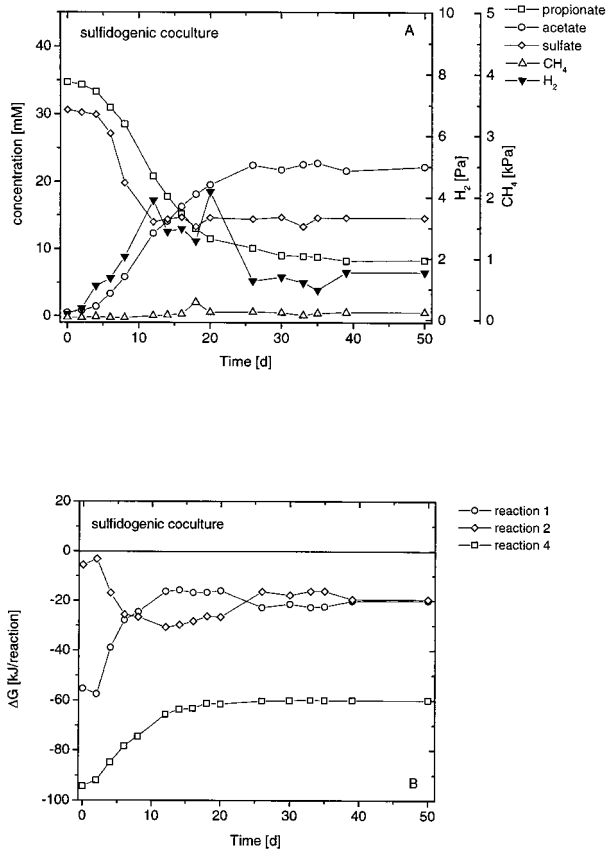


FIG. 3. Batch sulfidogenic coculture of *S. fumaroxidans* and *M. hungatei* growing on propionate plus sulfate. (A) Changes in propionate (□), acetate (○), hydrogen (▼), sulfate (◇), and methane (△). (B) Actual Gibbs free-energy changes of fermentative propionate oxidation (○), hydrogenotrophic methanogenesis (◇), and sulfidogenic propionate oxidation (□).

ues of propionate degradation under fermentative, syntrophic, and sulfidogenic conditions, as well as H_2-dependent methanogenesis, are shown in Fig. 2B and 3B for the syntrophic and sulfidogenic cocultures, respectively. *S. fumaroxidans* grown in a coculture with *M. hungatei* on propionate plus sulfate is referred to as a sulfidogenic coculture.

The μ_{max}, $Y_{Xtot\text{-}S}$, and carbon and electron recoveries were calculated for three batch cultures and are summarized in Table 2. The culture conditions, i.e., pure culture, syntrophic coculture, and sulfidogenic coculture, significantly affected the μ_{max} and $Y_{Xtot\text{-}S}$ of the cocultures. The highest μ_{max} value was found in the syntrophic cocultures, followed by the sulfidogenic coculture.

The calculated $Y_{MPOB\text{-}S}$ of *S. fumaroxidans* and $Y_{Mhun\text{-}P}$ of *M. hungatei* are presented in Table 3. The error associated with the calculation of the X_i of the propionate-degrading and methanogenic microorganisms calculated from the X_{tot}, the N_i, and the x_i was, in all cases, <20%. Furthermore, the q_{max} values for propionate were calculated from μ_{max} and $Y_{MPOB\text{-}S}$. *S. fumaroxidans* obtained the highest $Y_{MPOB\text{-}S}$ in the pure-culture incubation, followed by the syntrophic coculture and the sulfidogenic coculture (Table 3). *M. hungatei*, on the other hand, reached the highest $Y_{Mhun\text{-}P}$ in the sulfidogenic coculture rather than in the syntrophic coculture (Table 3). The highest q_{max} values were observed in the syntrophic coculture, followed by the sulfidogenic coculture and the pure culture (Table 3).

Chemostat experiments. Syntrophic cocultures of *S. fumaroxidans* and *M. hungatei* were grown in chemostats with or without a flushed headspace. The conversion of substrates to products was generally well balanced (C balance, 116 to 130% and e balance, 96 to 105%). The steady-state partial pressures of H_2 were 0.2 to 0.3 and 4.5 to 10 Pa in the flushed and nonflushed systems, respectively.

The actual Gibbs free energy of propionate fermentation (reaction 1 in Table 1) under steady-state conditions decreased linearly (becoming more negative) with increasing growth rate (Fig. 4A). Due to the lower H_2 partial pressure, the Gibbs free energy was more negative in the flushed system than in the nonflushed system. The ranges of Gibbs free energies available under steady-state conditions in the flushed and nonflushed systems were −35.0 to −37.8 and −6.3 to −11.5 kJ per mol of propionate, respectively. On the other hand, the actual Gibbs free energy of hydrogenotrophic methanogenesis (reaction 2 in Table 1) under steady-state conditions increased linearly with the growth rate (Fig. 4B). Due to the lower H_2 partial pressure, the Gibbs free energy was more positive in the flushed system than in the nonflushed system. The ranges of Gibbs free energies available under steady-state conditions in the flushed and nonflushed systems were −9.0 to −14.1 and −25.7 to −34.0 kJ per mol of CH_4, respectively.

X_{tot}, q_s, and q_{CH4} increased linearly with growth rates (data not shown). X_{tot} values were higher in the flushed system than in the nonflushed system. The opposite was true for the specific propionate conversion rates, which were the highest in the nonflushed system.

Growth yields of the total coculture and of each syntrophic partner were determined individually by the total dw, the relative cell number, and the specific cell masses. Total dw was determined by multiplying the measured protein concentration by the determined conversion factor (Table 2).

The average growth yields ($n = 4$) for the total coculture obtained in the flushed and nonflushed systems were 4.0 ± 0.5 and 2.2 ± 0.2 g (dw) mol of S^{-1}, respectively. These values were used to calculate Y_{tot}^{max} and $m_{s\text{-}tot}$ from the regression

TABLE 2. μ_{max}, $Y_{Xtot\text{-}S}$, C and e recoveries, and protein conversion factor[a] for batch cultures of *S. fumaroxidans* grown in a monoculture or in a coculture with *M. hungatei* on either propionate alone or propionate plus sulfate[b]

Culture	Substrate	$Y_{Xtot\text{-}S}$ (g [dw] mol^{-1})	μ_{max} (day^{-1})	C recovery[c] (%)	e recovery[c] (%)	Conversion factor
Monoculture	Propionate + SO_4^{2-}	1.24 (0.21)	0.020 (0.002)	103 (2)	95 (2)	3.0 (0.5)
Coculture	Propionate	1.57 (0.07)	0.208 (0.012)	113 (1)	99 (1)	2.5 (0.2)
Coculture	Propionate + SO_4^{2-}	1.28 (0.04)	0.089 (0.005)	107 (2)	97 (2)	2.3 (0.2)

[a] Conversion factor for protein to dw.
[b] Values in parentheses represent 1 standard deviation of the measured values ($n = 3$).
[c] For the calculation of C and e recovery, the total dw was calculated by multiplying the measured protein concentration by the determined conversion factor. $C_4H_{7.2}O_2N_{0.8}$ was used as the structural formula for biomass.

TABLE 3. $Y_{Mhun\text{-}P}$, $Y_{MPOB\text{-}S}$, q_{max}, actual ΔG, and $Y_{MPOB}^{\Delta G}$ for propionate-oxidizing *S. fumaroxidans* grown under three different batch conditions

Culture	Substrate	$Y_{MPOB\text{-}S}$ (g [dw] mol of S^{-1})	$Y_{Mhun\text{-}P}$ (g [dw] mol of P^{-1})	q_{max}[a] (mol of S [g [dw] d^{-1}]$^{-1}$)	ΔG[b] (kJ mol of S^{-1})	$Y_{MPOB}^{\Delta Gc}$ (g [dw] 70 kJ^{-1})
Monoculture	Propionate+SO_4^{2-}	1.24 (0.21)		0.016	−74.3 (2.4)	1.2
Coculture	Propionate	1.02 (0.04)	0.73 (0.07)	0.204	−12.6 (2.7)	5.7
Coculture	Propionate+SO_4^{2-}	0.82 (0.04)	17.7 (2.0)	0.109	−62.3 (1.7)	0.9

[a] $q_{max} = \mu_{max}/Y_{MPOB\text{-}S}$.
[b] Values are the actual Gibbs free-energy changes for the conversion of propionate by *S. fumaroxidans* (reaction 4 in Table 1), syntrophic coculture (reaction 1 in Table 1), and sulfidogenic coculture (reaction 4). The average actual Gibbs free energies between 11 and 29, 8 and 20, and 12 and 26 days, respectively (Fig. 1B, 2B, and 3B), are shown.
[c] Measured growth yields (see Table 2), normalized to an energy quantum of 70 kJ mol of S^{-1}, are shown.

parameters of the Pirt equation (equation 4) and are listed in Table 4.

The average growth yields ($n = 4$) for *S. fumaroxidans* obtained in the flushed and nonflushed systems were 2.7 ± 0.5 and 1.5 ± 0.1 g (dw) mol of S^{-1}, respectively. These values were used to calculate Y_{MPOB}^{max} and m_s from the regression parameters of the Pirt equation (Fig. 5A) and are listed in Table 4.

The average growth yields ($n = 4$) for *M. hungatei* were calculated by relating the increase in the biomass concentration to the amount of methane formed. The amount of methane formed was calculated by assuming that 0.75 mol of methane was formed per mol of propionate (Table 1, reaction 3). The growth yields of *M. hungatei* obtained in the flushed and nonflushed systems were 6.6 ± 0.6 and 4.6 ± 0.7 g (dw) mol of P^{-1}, respectively. These values were used to calculate Y_{Mhun}^{max} and m_s from the regression parameters of the Pirt equation (Fig. 5B) and are listed in Table 4.

D_s^{01}/r_{Ax}, calculated from the determined Y_{tot}^{max} of the chemostat cocultures of *S. fumaroxidans* and *M. hungatei*, in the flushed and nonflushed systems, were 56.1 and 167.6 kJ mol of C^{-1}, respectively. The D_s^{01}/r_{Ax} calculated from the determined Y_{MPOB}^{max} of *S. fumaroxidans* in the flushed and nonflushed systems were −320.7 and −705.7 kJ mol of C^{-1}, respectively.

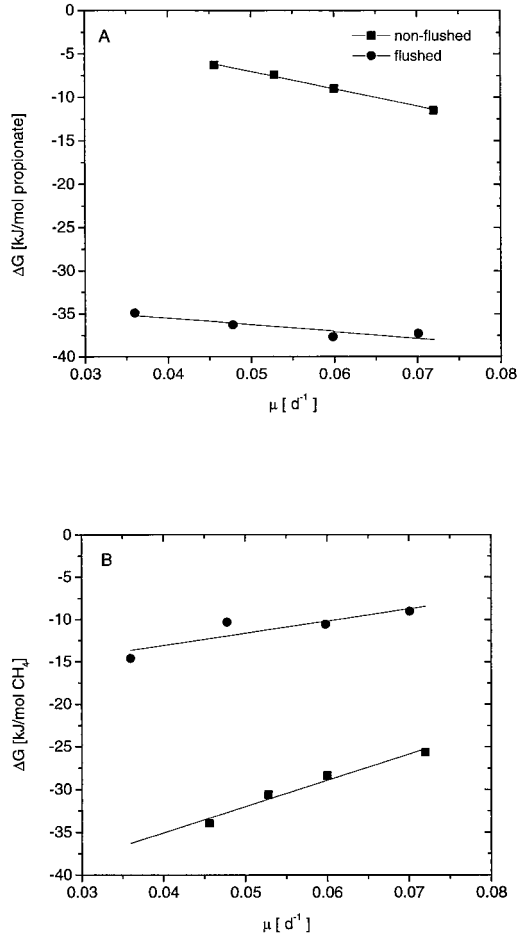


FIG. 4. Actual Gibbs free-energy changes available for *S. fumaroxidans* (A) and *M. hungatei* (B) determined during syntrophic growth in propionate-limited chemostat cocultures with a flushed (●) or nonflushed (■) gas headspace.

DISCUSSION

Energetics of syntrophic propionate oxidation. The Gibbs free energy available for propionate-oxidizing *S. fumaroxidans* was found to be higher in the flushed (−35 to −38 kJ mol of propionate^{-1}) than in the nonflushed (−6 to −12 kJ mol of propionate^{-1}) chemostat cocultures. A possible disequilibrium of H_2 between the liquid and gas phases would have resulted in even higher Gibbs free energies (less exergonic) than indicated by the values above. Disregarding this potential bias, the apparent Gibbs free-energy change in the flushed system is sufficient to generate not more than 1/2 mol of ATP, while in the nonflushed system less than 1/11 to 1/7 mol of ATP can be generated. The possible ATP generation based on the Gibbs free-energy change in the syntrophic batch cultures (−12.6 kJ mol of propionate^{-1}; Table 3) was similarly low as in the nonflushed chemostat. The Gibbs free-energy change in the flushed chemostat system is more than the minimum energy quantum necessary to sustain microbial life (−23 kJ mole of substrate^{-1} ≅ 1/3 mol of ATP), but this is not the case in the nonflushed chemostat and in the batch culture. Nevertheless, our experiments show that microbial growth was sustained even in the nonflushed system. Hence, the available Gibbs free energy was apparently sufficient. Relatively small free-energy changes during the degradation of propionate have also been reported for different methanogenic environments (5, 19, 26, 46). These observations raise the question of how *S. fumaroxidans* and other syntrophic propionate-oxidizing bacteria manage to exploit the little Gibbs free energy available for the generation of ATP. More research is required to answer this question.

Growth parameters. Measured values of growth parameters obtained in batch experiments depended on the type of culture and the overall propionate-consuming reaction. *S. fumaroxidans* had the highest $Y_{MPOB\text{-}S}$ but the lowest μ_{max} when grown as a pure culture (propionate plus sulfate). The values of $Y_{MPOB\text{-}S}$ and μ_{max} (Table 2) we obtained correspond well to the

TABLE 4. Y_{max} and m_s of *S. fumaroxidans* cocultured with *M. hungatei* in two different chemostat set-ups

Chemostat flushing	Y_{tot}^{max} (g mol of S^{-1})[a]	$m_{s\text{-}tot}$ (mmol of S g [dw]$^{-1}$ h^{-1})[a]	Y_{MPOB}^{max} (g mol of S^{-1})[b]	$m_{s\text{-}MPOB}$ (mmol of S g [dw]$^{-1}$ h^{-1})[b]	Y_{Mhun}^{max} (g mol of P^{-1})[c]	$m_{s\text{-}Mhun}$ (mmol of S g [dw]$^{-1}$ h^{-1})[c]
Yes	6.7	0.29	5.7	0.42	7.8	0.05
No	3.1	0.21	2.6	0.63	7.6	0.21

[a] Y^{max} and m_s for the coculture (data not shown).
[b] Y^{max} and m_s for *S. fumaroxidans* calculated from Fig. 5A.
[c] Y^{max} and m_s for *M. hungatei* calculated from Fig. 5B.

previously reported values of 1.5 g (dw) mol of S^{-1} and 0.024 day^{-1} (40), respectively. The μ_{max} values reported for *S. fumaroxidans* cocultured with *M. hungatei* on propionate was reported to be 0.17 day^{-1} (32). We obtained a value of 0.21 ± 0.01 day^{-1} for μ_{max}, which is in the same range as the reported value. Growth yields of *S. fumaroxidans* in syntrophic or sulfidogenic cocultures are not reported in the literature. The growth yields we calculated for *S. fumaroxidans* were 1.02 ± 0.04 and 0.82 ± 0.04 g (dw) mol of S^{-1}, respectively. Our results showed that the calculated and reported yields of *S. fumaroxidans* grown as a pure culture were higher than the values obtained in syntrophic and sulfidogenic cocultures. The reason for the lower growth yields in the cocultures can be explained by the fact that the available energy has to be shared between the two syntrophic partners.

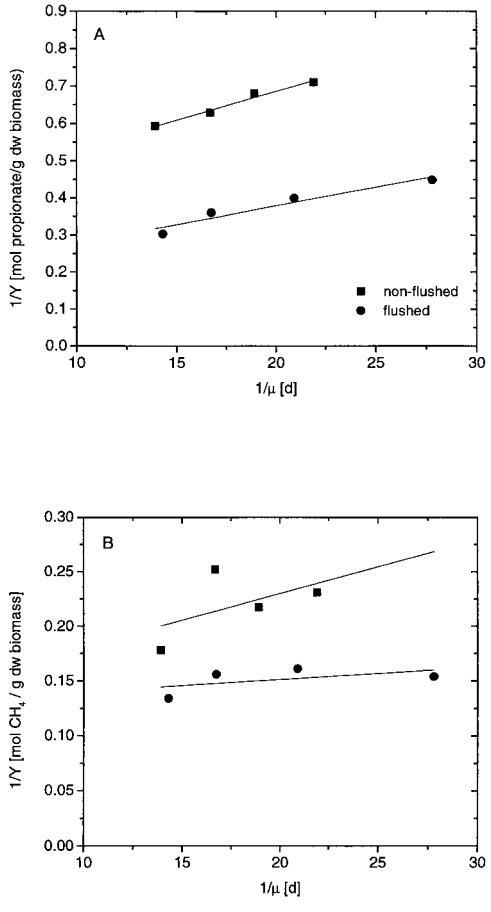


FIG. 5. Reciprocal plots of growth yields ($1/Y$) versus growth rates ($1/\mu$) of *S. fumaroxidans* (A) ($1/Y_{MPOB}$) and *M. hungatei* (B) ($1/Y_{Mhun}$) in propionate-limited chemostat cocultures with a flushed (●) or nonflushed (■) gas headspace.

The Y_{MPOB}^{max} and the m_s for *S. fumaroxidans* and *M. hungatei* cocultured on propionate were determined in chemostat cultures (Table 4). To our knowledge, these are the first data obtained for a syntrophic propionate-oxidizing bacterium. The values are comparable to those determined for *Pelobacter acetylenicus* growing on ethanol syntrophically with different H_2-consuming anaerobes (31).

Maintenance energy. Tijhuis et al. (38) showed that the maintenance requirements of microorganisms can be described on the basis of m_E, which theoretically should only be a factor of temperature. Thus, the theoretical m_E values at 28, 30, and 37°C are 4.4 ± 1.4, 5.2 ± 1.7 and 9.8 ± 3.1 kJ mol of biomass C^{-1} h^{-1}, respectively (38). We calculated the parameter m_E from literature data on an ethanol-oxidizing syntrophic coculture on several anaerobic pure cultures and included the data obtained from the chemostat cocultures of *S. fumaroxidans* and *M. hungatei* (Table 5). Interestingly, these m_E values were usually lower than the theoretical values, some by more than 1 order of magnitude.

Heijnen and VanDijken (13) pointed out that to obtain meaningful values, the ΔG_{av} should be calculated from the actual concentrations of reactants and products and that the use of ΔG_{av}^{01}, as done for calculation of the data in Table 5, is only a compromise if actual concentrations are not available. Therefore, we also calculated m_E values from the actual ΔG_{av} values measured for propionate oxidation by *S. fumaroxidans* (Fig. 4A) and H_2-CO_2-dependent methanogenesis by *M. hungatei* (Fig. 4B) in the chemostat experiments, using the species-specific m_s values (Table 6). Again, however, the m_E values of *S. fumaroxidans* and *M. hungatei* were more than 1 order of magnitude lower than the theoretical values expected from the equation of Tijhuis et al. (38). Obviously, the maintenance energies calculated from our chemostat experiments and from earlier experiments with anaerobic cultures are not consistent with the pertinent theory, suggesting that microorganisms, at least anaerobes, are able to grow at maintenance energies lower than those theoretically predicted. Tijhuis et al. (38) have concluded that (i) the electron acceptor, (ii) different C sources, (iii) the type of organism, and (iv) the use of mixed sludges versus pure cultures have no effect on m_E. However, this conclusion was based on limited culture data. In particular, syntrophic cultures have not been included. In addition, Heijnen and VanDijken (13) cautioned that the derived theoretical relationships give only a first approximation based on thermodynamics and that modifications may be necessary if mechanistic details become available, e.g., microbes exploiting the available Gibbs free energy by using different metabolic pathways with different energetic efficiencies. There are even differences among the various physiological groups of anaerobic microbes in whether they depend on changes in reaction enthalpy or reaction entropy (42). For example, H_2-CO_2-dependent methanogenesis depends mainly on the enthalpy change and is retarded by the entropy change whereas anaerobes, syntrophs in particular, depend mainly on the entropy change

TABLE 5. m_E determined for different anaerobic monocultures and cocultures grown under different conditions using the measured m_s and the ΔG_{av}^{01} of the overall catabolic reaction

Microorganism	Temp (°C)	Substrate	ΔG_{av}^{01} (kJ e-mol^{-1})	γ_D	m_D (mol of [C] mol of C^{-1} h^{-1})	m_E (kJ mol of C^{-1} h^{-1})	Reference(s)
S. fumaroxidans cocultured with *M. hungatei*							
Flushed chemostat	37	Propionate	1.86[a]	4.67	0.023	0.20	This study
Nonflushed chemostat	37	Propionate	1.86[a]	4.67	0.016	0.14	This study
P. acetylenicus cocultured with H_2 oxidizer:							
Acetobacterium woodii	28	Ethanol	3.54[b]	6	0.100	2.12	30, 31
Methanobacterium bryantii	28	Ethanol	4.87[c]	6	0.063	1.84	30, 31
Desulfovibrio desulfuricans	28	Ethanol	5.56[d]	6	0.038	1.27	30, 31
Pure-culture studies							
Desulfovibrio propionicus	28	Ethanol	5.56[d]	6	0.047	1.56	35
Desulfovibrio vulgaris Marburg	28	Ethanol	5.56[d]	6	0.224	7.47	35
Pelobacter propionicus	28	Ethanol	3.48[e]	6	0.078	1.63	35
Acetobacterium carbinolicum	28	Ethanol	3.54[b]	6	0.172	3.65	35
Acetobacterium woodii	30	Lactate	4.67[f]	4	0.006	0.12	23
Acetobacterium woodii	30	H_2	12.7[g]	2	0.042	1.06	23

[a] Reaction 3 in Table 1.
[b] Reaction: $2CH_3CH_2OH + 2HCO_3^- \rightarrow 3CH_3COO^- + H^+ + 2H_2O$.
[c] Reaction: $2CH_3CH_2OH + HCO_3^- \rightarrow 2CH_3COO^- + H^+ + CH_4 + H_2O$.
[d] Reaction: $2CH_3CH_2OH + SO_4^{2-} \rightarrow 2CH_3COO^- + H^+ + HS^- + 2H_2O$.
[e] Reaction: $8CH_3CH_2OH + 6HCO_3^- \rightarrow 5CH_3COO^- + 4CH_3CH_2COO^- + 3H^+ + 8H_2O$.
[f] Reaction: $CH_3CH_2OCOO^- \rightarrow 1.5CH_3COO^- + 0.5H^+$.
[g] Reaction: $4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$.

(42). Apparently, more research is necessary to elucidate the theoretical background of maintenance energy in fastidious anaerobic bacteria.

Gibbs free-energy dissipation per mol of biomass C. Heijnen and VanDijken (13) showed that D_s^{01}/r_{Ax} can be regarded as a simple thermodynamic measure of the amount of biochemical "work" required to convert a carbon source into biomass and can be used to characterize chemotrophic microbial growth. We calculated D_s^{01}/r_{Ax} from the maximum growth yields determined for the syntrophic chemostat cocultures of *S. fumaroxidans* and *M. hungatei*. The calculated D_s^{01}/r_{Ax} values for the flushed and nonflushed systems were 56.1 and 167.6 kJ mol of biomass C^{-1}, respectively. These values are at the lower end of the range of values (150 to 3,500 kJ mol of biomass C^{-1}) observed for various modes of chemotrophic growth (13). Furthermore, we calculated D_s^{01}/r_{Ax} for *S. fumaroxidans* alone and obtained values of −320.7 and −705.7 kJ mol of biomass C^{-1}, respectively, for the flushed and nonflushed systems. These negative values are not realistic and can be explained by the fact that the D_s^{01} is endergonic when using the standard Gibbs energies of formation for the reactants and products under standard conditions.

Therefore, we also calculated theoretical growth yields from a theoretical D_s^{01}/r_{Ax}, which is 426 kJ mol of biomass C^{-1} for propionate. For the syntrophic coculture (equation 7), we calculated a Y_{tot}^{max} of 4.4 g mol of S^{-1}, i.e., similar to the actually observed values (Table 4). However, for *S. fumaroxidans* alone (equation 8), we obtained a negative Y_{MPOB}^{max} value, indicating that the theoretical D_s^{01}/r_{Ax} must be too low. Only when we assumed a D_s^{01}/r_{Ax} of >622 kJ mol of biomass C^{-1} did the calculated Y_{MPOB}^{max} become positive. When assuming a D_s^{01}/r_{Ax} of 3,500 kJ mol of biomass C^{-1}, we calculated a Y_{MPOB}^{max} of 1.95 g mol of S^{-1}, i.e., similar to the actually observed values (Table 4). A D_s^{01}/r_{Ax} as high as 3,500 kJ mol of biomass C^{-1} is usually observed in chemolithoautotrophic bacteria that use CO_2 as a carbon source and require the occurrence of reversed electron transport, e.g., nitrifiers and thiobacilli. Our data indicate that syntrophic propionate oxidation by *S. fumaroxidans* falls into the same category of Gibbs free-energy dissipation.

TABLE 6. m_E determined from the m_s and the actual ΔG_{av} measured for *S. fumaroxidans* and *M. hungatei* grown in syntrophic cocultures with two different chemostat setups

Microorganism and chemostat condition[a]	Substrate	ΔG_{av} (kJ mol of e^{-1})	γ_D	m_D (mol of [C] mol of C^{-1} h^{-1})	m_E (kJ mol of C^{-1} h^{-1})
S. fumaroxidans					
Flushed	Propionate	2.59[b]	4.67	0.033	0.40
Nonflushed	Propionate	0.61[b]	4.67	0.049	0.14
M. hungatei					
Flushed	H_2	1.44[c]	2	0.005	0.01
Nonflushed	H_2	3.74[c]	2	0.022	2.16

[a] The temperature was 37°C in all cases.
[b] Reaction 1, Fig. 4A.
[c] Reaction 2, Fig. 4B.

Energetic efficiency of growth. We were able to determine both the growth yield of *S. fumaroxidans* and the Gibbs free energy available by oxidation of propionate (Table 3). Although the net generation of ATP during the degradation of propionate is not clear, we were able to use the determined yields and Gibbs free-energy values to estimate a proxy (i.e., $Y_{MPOB}^{\Delta G}$) for Y_{ATP}, i.e., the biomass synthesized from 1 mol of ATP generated. Under physiological conditions, an average Gibbs free energy of 70 kJ is needed for the irreversible synthesis of 1 mol of ATP. Therefore, $Y_{MPOB}^{\Delta G}$ was calculated by normalizing the measured $Y_{MPOB\text{-}S}$ to an energy quantum of 70 kJ mol^{-1} (16, 31). The results are shown in Table 3.

The $Y_{MPOB}^{\Delta G}$ values calculated for *S. fumaroxidans* grown on propionate plus sulfate in either a monoculture and a sulfidogenic coculture were 1.2 and 0.9 g (dw) 70 kJ^{-1}, respectively. These values are much lower than the values of 5 to 12 g mol of ATP^{-1} reported by Stouthamer (34) for fermenting

bacteria. The $Y_{MPOB}^{\Delta G}$ value for *S. fumaroxidans* grown on propionate in a syntrophic coculture was 5.7 g (dw) 70 kJ^{-1}. The $Y_{MPOB}^{\Delta G}$ values calculated for flushed and nonflushed chemostat cocultures grown on propionate were 4.5 to 6.2 and 10.3 to 15.7 g (dw) 70 kJ^{-1}, respectively. These values suggest that the energy efficiency in syntrophic cocultures was much higher than in sulfidogenic monocultures and cocultures.

A possible reason for the energetic inefficiency of sulfidogenic oxidation of propionate may be found in the mechanism of the reduction of sulfate to sulfide. With sulfate as an electron acceptor, substrate level phosphorylation might be more effective for the syntrophic propionate oxidizer but sulfate has to be activated first. This activation costs the cell 2 mol of ATP per mol of sulfate. Additionally, the cell losses another 1/3 mol of ATP during the transport of sulfate across the microbial membrane. So, the activation and transport of sulfate costs the cell 2 1/3 mol of ATP per mol of sulfate (47). This amount of energy cannot be generated at substrate level phosphorylation alone (maximum of 1 1/3 mol of ATP per mol of sulfate consumed). Thus, it is most likely that *S. fumaroxidans* associates the reduction of sulfate with an electron transport chain allowing chemiosmotic ATP synthesis. If this process operates inefficiently, it may explain the observed low energy efficiency for growth. Furthermore, this hypothesis could explain why syntrophic propionate-oxidizing bacteria are outcompeted by propionate-oxidizing sulfate reducers. However, to our knowledge, no data concerning the energy efficiency for growth on propionate and sulfate for propionate-oxidizing sulfate reducers is available. Thus, there is no direct evidence to support these hypotheses.

Conclusion. It is postulated that the small Gibbs free-energy changes observed during the degradation of propionate in different methanogenic environments are sufficient to sustain microbial growth. However, the Gibbs free-energy changes observed are much lower than those theoretically predicted. Furthermore, it is assumed that the calculated low energetic efficiency of *S. fumaroxidans* during growth on propionate plus sulfate can be due to an inefficient electron transport chain involved in chemiosmotic energy conservation. The observed growth yields further suggest that *S. fumaroxidans* requires a relatively large Gibbs free-energy dissipation for biomass synthesis, similar to that typically observed for chemolithoautotrophic bacteria with reversed electron transport.

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