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Evidence of reversed electron transport in syntrophic butyrate or benzoate oxidation by *Syntrophomonas wolfei* and *Syntrophus buswellii*

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Abstract *Syntrophomonas wolfei* and *Syntrophus buswellii* were grown with butyrate or benzoate in a defined binary coculture with *Methanospirillum hungatei*. Both strains also grew independent of the partner bacteria with crotonate as substrate. Localization of enzymes involved in butyrate oxidation by *S. wolfei* revealed that ATP synthase, hydrogenase, and butyryl-CoA dehydrogenase were at least partially membrane-associated whereas 3-hydroxybutyryl-CoA dehydrogenase and crotonase were entirely cytoplasmic. Inhibition experiments with copper chloride indicated that hydrogenase faced the outer surface of the cytoplasmic membrane. Suspensions of butyrate- or benzoate-grown cells of either strain accumulated hydrogen during oxidation of butyrate or benzoate to a low concentration that was thermodynamically in equilibrium with calculated reaction energetics. The protonophore carbonylcyanide *m*-chlorophenyl-hydrazone (CCCP) and the proton-translocating ATPase inhibitor *N,N'*dicyclohexylcarbodiimide (DCCD) both specifically inhibited hydrogen formation from butyrate or benzoate at low concentrations, whereas hydrogen formation from crotonate was not affected. A menaquinone was extracted from cells of *S. wolfei* and *S. buswellii* grown syntrophically in a binary methanogenic culture. The results indicate that a proton-potential-driven process is involved in hydrogen release from butyrate or benzoate oxidation.

Key words Syntrophic oxidation · Butyrate · Benzoate H_2 formation · *Syntrophomonas wolfei* · *Syntrophus buswellii*

Abbreviations *BES* Bromoethanesulfonate · *CCCP* Carbonyl cyanide-*m*-chlorophenyl-hydrazone · *DCCD* *N,N'*dicyclohexylcarbodiimide · *DCPIP* Dichlorophenol indophenol · *PMS* Phenazine methosulfate

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Introduction

Fermentation of butyrate or benzoate to acetate (CO_2) and hydrogen are endergonic reactions under standard conditions [all calculations are based on Thauer et al. (1977), with CO_2 and H_2 in the gaseous state]:

$$\text{butyrate}^- + 2\ H_2O \rightarrow 2\ \text{acetate}^- + H^+ + 2\ H_2 \qquad \Delta G^{0'} = +48.3\ \text{kJ per mol butyrate}$$

$$\text{benzoate}^- + 6\ H_2O \rightarrow 3\ \text{acetate}^- + 2\ H^+ + CO_2 + 3\ H_2 \qquad \Delta G^{0'} = +49.5\ \text{kJ per mol benzoate.}$$

Degradation of both compounds becomes feasible only at low hydrogen partial pressure (10^{-4}–10^{-5} atm; Schink 1992), which can be maintained by hydrogen-oxidizing anaerobes such as methanogenic bacteria (Zehnder 1978; Dolfing 1988). The pathways of butyrate and benzoate degradation in these bacteria have been at least tentatively elucidated (see Schink 1992). The energetically most difficult electron transfer steps are the oxidations of the saturated acid esters butyryl-CoA or glutaryl-CoA to the respective unsaturated compounds. The electrons released in the butyryl-CoA dehydrogenase reaction ($E_0' = -125$ mV; Gustafson et al. 1986) and glutaryl-CoA dehydrogenase, for which a similar redox potential is assumed, are used to reduce protons to molecular hydrogen ($E_0' = -414$ mV). Even at 10^{-4} atm hydrogen, the redox potential of the couple $2\ H^+/H_2$ is still –295 mV and is much lower than that of the electron donor. It has been hypothesized, therefore, that part of the ATP gained by substrate level phosphorylation during butyrate oxidation has to be spent in a reversed electron transport step to shift these electrons to a lower redox potential (Thauer and Morris 1984). Similar problems arise with oxidation of saturated intermediates in syntrophic benzoate degradation, and involvement of reversed electron transport in this oxidation also has been postulated (Schink 1992). However, experimental evidence of such energy-driven processes in syntrophic butyrate or benzoate oxidation has never been provided.

Syntrophomonas wolfei was the first syntrophic butyrate-degrader isolated in a binary methanogenic cocul-

ture (McInerney et al. 1981). The pathway of butyrate oxidation has been elucidated by enzyme measurements in cell-free extracts of this culture (Wofford et al. 1986). The only syntrophic culture isolated with benzoate as substrate, *Syntrophus buswellii*, could be obtained in a binary mixed culture only with *Desulfovibrio* sp., or in a ternary methanogenic coculture with *Methanospirillum hungatei* and *Desulfovibrio* sp. (Mountfort et al. 1984).

The aim of the present study was to check for a possible involvement of reversed electron transport in syntrophic oxidation of butyrate and benzoate, and its possible association with membrane-bound energy-transducing components. Such studies required defined binary cocultures of both syntrophic bacteria with methanogens as the sole partner bacteria.

Materials and methods

Organisms and cultivation

The binary methanogenic coculture *Syntrophomonas wolfei* (DSM 2245 B) and the ternary methanogenic coculture *Syntrophus buswellii*, which also contained *Desulfovibrio* sp. (DSM 2612 B), were obtained from the Deutsche Sammlung von Mikroorganismen GmbH (Braunschweig, Germany). *Methanospirillum hungatei* strain SK was kindly provided by Prof. Dr. F. Widdel (Bremen, Germany).

All procedures for cultivation were as previously described (Widdel and Pfennig 1981). The mineral medium contained 0.5 g NaCl and 0.4 g $MgCl_2 \times 6\ H_2O$ per l, was buffered with 30 m*M* bicarbonate, and contained 1 m*M* sodium sulfide as reducing agent, trace element solution SL 10 (Widdel et al. 1983), selenite-tungstate solution (Tschech and Pfennig 1984), and a 7-vitamin solution (Pfennig 1978). The pH was adjusted to 7.2–7.4. Addition of small amounts (50–100 μ*M*) of sodium dithionite helped to shorten growth lags. Acetate, butyrate, crotonate, and benzoate were added from autoclaved neutral 1.0 *M* stock solutions. *M. hungatei* strain SK was grown in medium containing 5 m*M* acetate under H_2/CO_2 (80/20). For growth of *S. wolfei*, 0.05% (w/v) yeast extract was added to the medium. Cultures (1 l) were grown in 1200-ml infusion bottles under N_2/CO_2 (90/10) with 20 m*M* butyrate, 10 m*M* benzoate, or 10 m*M* crotonate. Growth at 28° C was followed measuring turbidity at 578 nm.

Preparation of cell suspensions

Cultures at the end of the logarithmic growth phase were transferred into oxygen-free centrifuge vials in an anoxic chamber (Coy, Ann Arbor, Mich., USA) and centrifuged at $8000 \times g$ for 30 min. Cells were suspended in 50 m*M* Mops/KOH buffer (pH 7.0) containing 5 m*M* $MgCl_2$, and were transferred into 120-ml infusion bottles. Cell suspensions in the infusion bottles were washed twice under anoxic conditions in the same buffer by centrifugation (20 min, $2100 \times g$). Cell suspensions (OD_{578} = 8–10) were stored under N_2 on ice.

Preparation of cell-free extracts and membrane fractions

Cells were harvested as described above, washed twice in anoxic potassium phosphate buffer (50 m*M*, pH 7.2) containing 5 m*M* $MgCl_2$, and resuspended in 3 ml of the same buffer. The suspension was passed 2–3 times through a French-pressure cell (140 MPa). Cell debris and intact cells were removed by centrifugation ($5000 \times g$, 15 min). The supernatant (crude cell extract) was centrifuged under anoxic conditions ($100{,}000 \times g$, 1 h). The $100{,}000 \times g$ supernatant (soluble fraction) was removed and stored anoxically on ice. The pellet (membrane fraction) was washed once in potassium phosphate buffer (50 m*M*, pH 7.2, with 5 m*M* $MgCl_2$) and resuspended in the same volume of buffer as the original crude extract.

Selective cell lysis with mutanolysin or lysozyme

Mutanolysin, a bacteriolytical enzyme from *Streptomyces globiformis* (Yokogawa et al. 1974), was used at activities of 50–150 U/mg protein in 100 m*M* anoxic potassium phosphate buffer (pH 6.2). Cells were incubated for 30 min at 37° C. Cell lysis was checked microscopically and by measuring enzyme activities in the supernatant after removal of intact cells by centrifugation ($5000 \times g$, 15 min). Selectivity of lysis was checked by treatment of a pure culture of *M. hungatei* grown with H_2/CO_2 under the same conditions. Fluorimetric determination of the methanogenic cofactor F_{420} was used as an indicator of contamination by cytoplasmic components of the methanogenic partner bacterium, using an Eppendorf 1101 *M* photometer with fluorescence capability (excitation wavelengths 405 and 436 nm). Additional controls were run with cell extracts prepared by French-press treatment (3 times, 136 MPa). Lysozyme treatment was performed anoxically in Tris/HCl buffer (10 m*M*, pH 8.0) containing 0.5 mg lysozyme and 0.4 mg EDTA per ml (2.5–5 μg lysozyme per mg protein). Suspensions were incubated at 37° C for 30 min and cell lysis was checked microscopically.

Enzyme measurements

All enzyme measurements were performed at 25° C in 1 cm cuvettes under a N_2 atmosphere using a spectrophotometer model 100-40 (Hitachi, Tokyo, Japan). Additions were made with microliter syringes.

Butyryl-CoA dehydrogenase (EC 1.3.99.3; modified after acyl-CoA dehydrogenase, Bergmeyer 1974) was measured with dichlorophenol indophenol (DCPIP) as electron acceptor in the presence of phenazine methosulfate (PMS) as intermediary electron carrier. The assay mixture contained 50 m*M* potassium phosphate buffer (pH 7.5), 0.2 m*M* DCPIP (ε_{600} = 20.0 $mM^{-1}\ cm^{-1}$), 0.1 m*M* PMS, and 0.1 m*M* butyryl-CoA.

Glutaryl-CoA dehydrogenase (EC 1.3.99.7) was determined in a way similar to succinate dehydrogenase (Stams et al. 1984) with $K_3Fe(CN)_6$ (ε_{420} = 0.9 $mM^{-1}\ cm^{-1}$) as electron acceptor in the presence of PMS. The assay mixture contained 50 m*M* potassium phosphate buffer (pH 7.5), 1 m*M* $K_3Fe(CN)_6$, o.1 m*M* PMS, and 0.05 m*M* glutaryl-CoA.

3-Hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) was determined following decrease of NADH at 365 nm in the presence of *S*-acetoacetyl-CoA (Bergmeyer 1974). The assay mixture contained 12.5 m*M* sodium pyrophosphate buffer (pH 7.3), 0.25 m*M* NADH, and 1 m*M* *S*-acetoacetyl-CoA.

Hydrogenase (EC 1.12.1.2) activity was measured following benzyl viologen (ε_{578} = 8.65 $mM^{-1}\ cm^{-1}$) reduction with hydrogen (Diekert and Thauer 1978).

ATPase (EC 3.6.1.3) activity was determined following NADH oxidation in the presence of ATP, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase (modified after Vogel and Steinhart 1976). The assay mixture contained 100 m*M* potassium phosphate buffer (pH 7.6), 5 m*M* $MgCl_2$, 1 m*M* NADH, 2 m*M* phosphoenolpyruvate, 5 U pyruvate kinase, and 20 U lactate dehydrogenase. The reaction was started by addition of 5 m*M* ATP.

Hydrogen release experiments

Assays were performed at 37° C in N_2-flushed 4-ml vials closed with butyl rubber septa. Fresh cell suspension (1 ml) was added by syringe. The assay mixture contained 5 m*M* bromoethanesulfonate (BES) to inhibit methanogenesis. Before the reaction was started

by addition of Na-butyrate, Na-benzoate, or Na-crotonate (10 m*M* final concentration), background hydrogen formation was followed for 10–30 min until a constant level was reached. For tests with DCCD, cell suspensions were preincubated for 15 min with an ethanolic solution of the inhibitor (25 nmol per mg protein). Controls were performed with pure ethanol. CCCP (10 nmol per mg protein) was added to the reaction mixture when hydrogen accumulation had already started.

Localization of hydrogenase activity through copper inhibition

Cell suspensions (OD_{578} approx. 3.0) were prepared as described above, preincubated anoxically with 1 m*M* $CuCl_2$ for 10–15 min, and then used for hydrogenase assay (after Cypionka and Dilling 1986). Similar experiments with crude French-press extract served as controls.

Cytochromes and quinones

Cytochromes were determined in cell-free extracts and membrane preparations were obtained by ultracentrifugation as described above. Redox difference spectra (dithionite-reduced minus air-oxidized) were measured with a Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland).

Quinones were extracted from dry cells (Collins 1985) with petrol ether : methanol (2 : 1 v/v) as solvent. The purified quinones were dissolved in isopropanol and examined by high performance liquid chromatography (Kroppenstedt 1985).

Chemical analyses

Fatty acids and crotonate were assayed by gas chromatography as described previously (Platen and Schink 1987). Hydrogen concentrations ($\leq 10^{-3}$ bar) were determined by gas chromatography with a Mercury Reduction Gas Detector (Wolters, Düsseldorf, Germany). Gases were separated at room temperature on a 1.8 m × 2 mm column packed with 5 Å molecular sieve (Serva, Heidelberg). Protein was quantified by a micro assay after Bradford (1976) using bovine serum albumin as standard.

Chemicals

All chemicals were of reagent grade quality and were obtained from Fluka (Neu-Ulm), Sigma (Deisenhofen), and Merck (Darmstadt). Biochemicals were from Boehringer (Mannheim), and Sigma (Deisenhofen). Mutanolysin was obtained from Sigma.

Results

Growth of *Syntrophus buswellii* in defined mineral medium

S. buswellii grew with benzoate in defined mineral medium. After repeated transfer into this medium, the contaminating *Desulfovibrio* sp. was diluted out. Since *S. buswellii* did not grow in agar shakes, the culture was diluted stepwise in liquid medium containing *M. hungatei*. Purity of this binary coculture was checked by growth experiments in medium containing 0.05% yeast extract, 10 m*M* benzoate, 10 m*M* sulfate, and 5 m*M* BES. In this medium, no growth occurred after 6 weeks of incubation. The binary methanogenic coculture grew with 10 m*M* benzoate at 28°C with a doubling time of 7.2 days (μ = 0.096 $days^{-1}$) (Fig. 1). *S. buswellii* also grew independently of

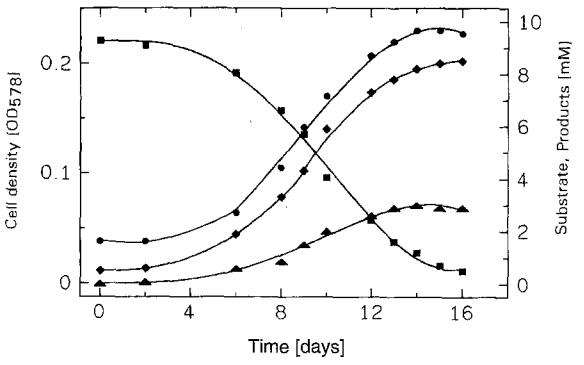

Fig. 1 Growth of *Syntrophus buswellii* with crotonate as substrate. The mineral medium contained 5 m*M* BES; the number of *Methanospirillum hungatei* cells was lower than 1% of total cell mass. Symbols: ● cell density, ■ crotonate, ▲ butyrate, ◆ acetate

Table 1 Release of butyryl-CoA dehydrogenase by mutanolysin treatment of the *Syntrophomonas wolfei/Methanospirillum hungatei* coculture and release of fluorescence by mutanolysin treatment of a pure culture of *M. hungatei*

Fraction	*S. wolfei/M. hungatei* Butyryl-CoA dehydrogenase		*M. hungatei* Relative fluoreschence[a]
	U[b]	Total (%)	Total (%)
French pass supernatant	0.175	100	100
Mutanolysin supernatant	0.132	75.4	15

[a] Relative fluorescence was determined to indicate the release of methanogenic cell material as described in 'Material and methods'
[b] µmol min^{-1} (mg $protein^{-1}$)

the partner bacteria with crotonate as substrate in the presence of 5 m*M* BES, at a growth rate of 0.14 day^{-1} (t_d = 4.8 days), according to the equation:

$$2\ \text{crotonate}^- + 2\ H_2O \rightarrow 2\ \text{acetate}^- + \text{butyrate}^- + H^+$$

The methanogenic partner could be diluted out on this substrate to less than 0.1% of bacterial biomass, but growth in a definite pure culture could not be obtained. Gentisate, hydroquinone, phthalate, pimelate, and cyclohexane carboxylate (each 5 m*M*) were not degraded by the binary methanogenic culture. Sulfate did not serve as alternative electron acceptor.

Selective lysis of *S. wolfei* cells with mutanolysin

Incubation of a cell suspension of the *S. wolfei/M. hungatei* coculture with mutanolysin resulted in complete lysis of the slightly curved *S. wolfei* cells. Cells of *M. hungatei* remained unaffected and were still motile. In the supernatant of the mutanolysin-treated cell suspension, 75% of total butyryl-CoA dehydrogenase activity of the crude French press extract was measured (Table 1). Longer incubation resulted in a decrease of the measurable enzyme activities. With a pure culture of *M. hungatei*, the supernatant was

Table 2 Distribution of butyryl-CoA dehydrogenase, hydrogenase, ATPase and 3-hydroxybutyryl-CoA dehydrogenase after fractionation of crude French press extract of *Syntrophomonas wolfei*

	Total activity		(%)[a]	
	Butyryl-CoA dehydrogenase	3-Hydroxybutyryl-CoA dehydrogenase	Hydrogenase	ATPase
Cell extract	100[a]	100[b]	100[c]	100[d]
Soluble fraction	65–70	80	83–95	39
Membrane fraction	13–18	1	8–15	55

[a] 100% = 0.19 μmol min^{-1} (mg protein)$^{-1}$
[b] 100% = 7.5 μmol min^{-1} (mg protein)$^{-1}$
[c] 100% = 0.23 μmol min^{-1} (mg protein)$^{-1}$
[d] 100% = 1.1 μmol min^{-1} (mg protein)$^{-1}$

Table 3 Distribution of butyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydrogenase, and hydrogenase after fractionation of crude extract obtained by mutanolysin lysis of *Syntrophomonas wolfei*

	Total activity		(%)
	Butyryl-CoA dehydrogenase	3-Hydroxybutyryl-CoA dehydrogenase	Hydrogenase
Cell extract	100	100	100
Soluble fraction	80	91	88
Membrane fraction	27	2	15

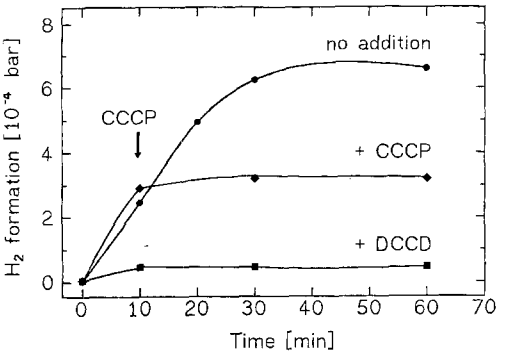

Fig. 2 H_2 formation in cell suspensions of *Syntrophomonas wolfei* with 20 m*M* butyrate and ● no inhibitor; ◆ after addition of CCCP (10 nmol per mg protein); and ■ after preincubation of the cell suspension with DCCD (15 min, 25 nmol per mg protein)

checked for an increase in fluorescence which would indicate release of the fluorescent methanogenic cofactor F_{420}; the amount of lysis of *M. hungatei* caused by mutanolysin treatment was low compared to the effect of mutanolysin on *S. wolfei* (Table 1). Cells of *S. buswellii* could not be disintegrated by lysis with mutanolysin or with lysozyme.

Localization of enzyme activities

Butyryl-CoA dehydrogenase, hydrogenase, 3-hydroxybutyryl-CoA dehydrogenase and ATPase were detected at catabolic activities in crude extract obtained by French-press treatment of the *S. wolfei/M. hungatei* coculture. NAD-dependent 3-hydroxybutyryl-CoA dehydrogenase and ATPase served as control enzymes. Butyryl-CoA dehydrogenase and hydrogenase activities were found to be partially associated with the membrane fraction after French-press treatment of crude extract (Table 2) or mutanolysin treatment (Table 3). On the other hand, 3-hydroxybutyryl-CoA dehydrogenase activity was found exclusively in the cytoplasmic fraction (Tables 2 and 3). The comparably high ATPase activity found in the cytoplasmic fraction could also be due to some extent to soluble phosphatases.

Localization of glutaryl-CoA dehydrogenase in *S. buswellii* was not possible. In crude extracts only low activities (0.066 μmol min^{-1} (mg protein)$^{-1}$) of the enzyme were detected, which could no longer be recovered after further fractionation.

Significant hydrogenase activity was measured in suspensions of intact cells of *S. wolfei/M. hungatei* or *S. buswellii/M. hungatei* cocultures [0.17 and 1.1 μmol min^{-1} (mg protein)$^{-1}$, respectively]. Anoxic incubation of cell suspensions with 1 m*M* $CuCl_2$ as a non-membrane-permeating hydrogenase inhibitor resulted in 90–95% inhibition of the measurable hydrogenase activity in both cocultures. Hydrogenase activity of a pure culture of *M. hungatei* could be measured only in cell extracts and was not detectable with intact cells.

Cytochromes and quinones

The binary methanogenic cultures of *S. buswellii* and *S. wolfei* were analyzed for the presence of cytochromes and quinones. No cytochromes could be detected in syntrophically grown cells of *S. buswellii*. *S. wolfei* grown with butyrate also did not contain cytochromes. Both strains contained a menaquinone that, for *S. wolfei*, was identified as menaquinone-7.

Hydrogen release experiments

Dense suspensions of butyrate-grown cells of *S. wolfei* formed hydrogen from butyrate at a rate of 2.7 nmol min^{-1} (mg protein)$^{-1}$; hydrogen accumulated to a partial pressure of 6.3×10^{-4} bar (Fig. 2). Hydrogen was formed from crotonate by crotonate-grown cells of *S. wolfei* at a rate of 0.6 nmol min^{-1} (mg protein)$^{-1}$, up to a pressure of 7×10^{-4}

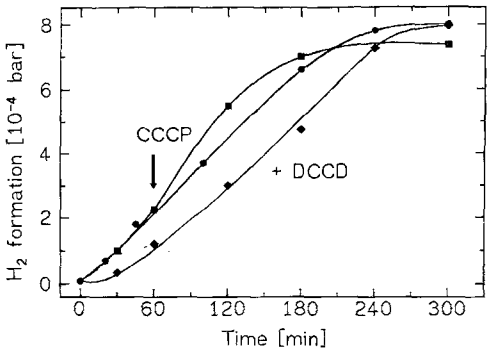

Fig. 3 H_2 formation in cell suspensions of *Syntrophomonas wolfei* with 20 m*M* crotonate and ● no inhibitor; ■ after addition of CCCP (10 nmol per mg protein); and ◆ after preincubation of the cell suspension with DCCD (15 min, 25 nmol per mg protein)

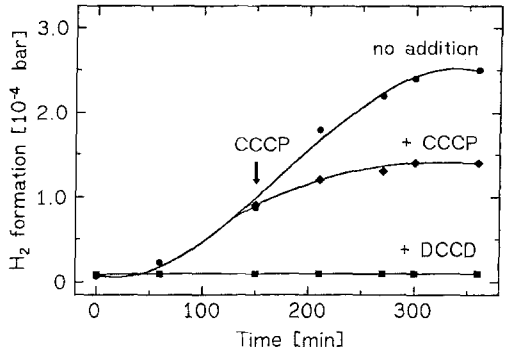

Fig. 4 H_2 formation in cell suspensions of *Syntrophus buswellii* with 10 m*M* benzoate and ● no inhibitor; ◆ after addition of CCCP (10 nmol per mg protein); and ■ after preincubation of the cell suspension with DCCD (15 min, 25 nmol per mg protein)

bar (Fig. 3). Addition of the protonophore CCCP (25 nmol per mg protein) completely inhibited hydrogen formation from butyrate, whereas CCCP (≤ 100 nmol per mg protein) had no effect on hydrogen formation from crotonate (Figs. 2 and 3). Similar results were obtained with DCCD; preincubation of butyrate-grown cell suspensions with DCCD (25 nmol per mg protein) resulted in complete inhibition of hydrogen formation (Fig. 2). Hydrogen formation from crotonate was not affected by DCCD up to 100 nmol per mg protein (Fig. 3). Hydrogen was not formed from crotonate by butyrate-grown cells of *S. wolfei* or from butyrate by crotonate-grown cells.

Cell suspensions of syntrophically grown cells of *S. buswellii* formed hydrogen from benzoate at a rate of 0.43 nmol min^{-1} (mg protein)$^{-1}$; hydrogen accumulated only up to a pressure of 2.5×10^{-4} bar (Fig. 4). Unfortunately, crotonate-grown cells of *S. buswellii* did not form significant amounts of hydrogen from crotonate, so a positive control experiment could not be performed. CCCP (10 nmol per mg protein) and DCCD (25 nmol per mg protein, 15 min preincubation) completely inhibited hydrogen formation from benzoate. Incubation of the cell suspension with the same amount of absolute ethanol as used in the DCCD and CCCP additions had no influence on the rate and amount of hydrogen formed by any cell suspension.

Dicussion

New physiological properties of *Syntrophus buswellii*

Isolation of a binary methanogenic coculture of the syntrophic benzoate-degrading culture *S. buswellii* made new physiological and biochemical studies possible. Crotonate is dismutated to butyrate and acetate in a manner similar to that observed with *Syntrophomonas wolfei* (Beaty and McInerney 1987). However, the lag phase for adaptation to the new substrate with benzoate-grown *S. buswellii* cells was not unusually long; with *S. wolfei* a long lag phase had been observed (Beaty and McInerney 1987). Butyrate-grown cells of *S. wolfei* do not possess crotonyl-CoA : acetate CoA transferase; therefore, the long lag phase was attributed to the selection of mutants able to activate crotonate (McInerney and Wofford 1992). It seems probable that benzoate-grown cells of *S. buswellii* have the ability to activate crotonate; however, this must be confirmed by enzyme measurements in cell-free extracts of the syntrophically grown culture. A recently isolated syntrophically benzoate-oxidizing bacterium can grow in pure culture with gentisate or hydroquinone as substrate (Szewzyk and Schink 1989; Gorny and Schink 1994).

Evidence of reversed electron transport

In syntrophic butyrate oxidation, electrons are released in two dehydrogenation steps, namely, butyryl-CoA dehydrogenase and 3-hydroxybutyryl-CoA dehydrogenase. The standard redox potential of the former was determined to be –125 mV (Gustafsson et al. 1986); for the latter the literature gives –231 mV (Lynen and Wieland 1955) or –266 mV (Krebs et al. 1962). We redetermined this value by equilibrium measurements with acetoacetyl-CoA, NADH, and 3-hydroxybutyryl-CoA dehydrogenase and found values of –239 ± 7 mV (results not shown). This value is close enough to the redox potential of the 2 H^+/H_2 couple at pH 7.0 and [H_2] at 10^{-4} atm (–295 mV) to allow the assumption that electron transfer from 3-hydroxybutyryl-CoA to protons does not require reversed electron transport; transfer from butyryl-CoA to protons, on the other hand, depends on such an energy-consuming step, which should be driven by, for example, the proton motive force. Such reversed electron transport processes in dissimilatory metabolism have been observed with cell suspensions of *Methanosarcina barkeri* (Bott and Thauer 1987) and *Desulfovibrio vulgaris* (Pankhania et al. 1988), as well as with membrane vesicles of the sulfur-reducing *Desulfuromonas acetoxidans* (Paulsen et al. 1986) or with membrane vesicles of a syntrophically glycolate-oxidizing bacterium (Friedrich and Schink 1993).

Energy-linked electron transport processes require the presence of specific electron transferring proteins and their association with the cytoplasmic membrane. In the present study, mutanolysin proved to be an excellent agent for selective and careful lysis of *S. wolfei* cells in a mixed

culture with *M. hungatei*. Seventy-five percent of the total activity of butyryl-CoA dehydrogenase of crude French-press extract was released by this method. Similar studies with lysozyme as the selective bacteriolytic agent resulted in a release of only 15.4% of total 3-hydroxybutyryl-CoA dehydrogenase activity (Wofford et al. 1986). Fluorescence measurements after mutanolysin treatment of *M. hungatei* indicated that the agent had only a slight lytic effect on the methanogenic partner.

Fractionation of the crude extract of *S. wolfei* obtained by mutanolysin lysis or French-press breakage indicated that hydrogenase and butyryl-CoA dehydrogenase activities were at least partially membrane-associated. There was a significant difference in localization between these enzymes and, for example, 3-hydroxybutyryl-CoA dehydrogenase, which was found entirely in the cytoplasm. Obviously, both enzymes are loosely attached to the membrane and may be connected with other transmembrane proteins.

Cells of *S. buswellii* were completely resistant to bacteriolytical agents, such as lysozyme or mutanolysin, and glutaryl-CoA dehydrogenase activity of crude French-press extracts was lost completely during further fractionation, thus precluding further studies on its localization.

Copper inhibition studies with suspensions of whole cells of *S. wolfei* and *S. buswellii* indicate that at least part of the hydrogenase activity of both strains is membrane-associated, and that it faces the outer side of the cytoplasmic membrane (Cypionka and Dilling 1986). The fact that in whole cells of a pure culture of *M. hungatei* grown with H_2/CO_2, a viologen-dependent hydrogenase activity could not be measured, whereas high activities were detected in crude extracts of this strain, indicates that the membrane of *M. hungatei* is not permeable to oxidized viologen dyes. This is in contrast to results obtained with *Desulfovibrio orientis* cells (Cypionka und Dilling 1986).

S. wolfei and *S. buswellii* both contained significant amounts of a menaquinone. This electron transfer component could be involved in reversed electron transport, but evidence is still lacking. Syntrophically grown cells of the *S. buswellii/M. hungatei* coculture were free of cytochromes. Cells of *S. wolfei* are reported to contain low amounts of a *c*-type cytochrome after growth with crotonate (McInerney and Wofford 1992), but we could not confirm this finding with our culture after syntrophic growth with butyrate. Perhaps the cytochrome plays a role only in crotonate metabolism by this strain.

Unfortunately, we were not able to prepare active inside-out vesicles for direct demonstration of a reversed electron transport either by mutanolysin or by careful French-press treatment of cell suspensions of *S. wolfei*. Therefore, direct evidence of a reversed electron transport in these bacteria had to be sought with suspensions of intact cells. Cell suspensions of *S. wolfei/M. hungatei* cocultures formed low, but significant amounts of hydrogen from butyrate; likewise, cell suspensions of *S. buswellii/M. hungatei* cocultures formed similar amounts of hydrogen from benzoate. Inhibition of this hydrogen formation by low amounts of CCCP or DCCD indicates that butyrate and benzoate conversion to acetate and hydrogen depends on an intact proton potential that is maintained by ATP hydrolysis. For *S. wolfei*, this effect can be confined specifically to butyryl-CoA oxidation because hydrogen formation from crotonate was not affected. Unfortunately, such a positive control experiment was not possible with crotonate-grown cells of *S. buswellii*, and nonspecific effects on, for example, substrate uptake, cannot therefore be ruled out with certainty.

Energetics

Cells of *S. wolfei* accumulated 6.3×10^{-4} atm or 120 μM hydrogen from 20 mM butyrate. According to the stoichiometry of butyrate oxidation by this bacterium,

$$\text{butyrate}^- + 2\ H_2O \rightarrow 2\ \text{acetate}^- + H^+ + 2\ H_2$$

acetate concentration should be 120 μM as well. With these data, the amount of energy in equilibrium with these concentrations can be calculated: $\Delta G' = -26.3$ kJ/mol reaction. Obviously, hydrogen and acetate accumulate to a concentration that corresponds thermodynamically with the equivalent of one-third of an ATP unit, which has been defined as the minimum amount of energy required to make ATP (Schink 1990, 1992). This is the amount of energy remaining from 1 ATP formed by substrate-level phosporylation in the acetate kinase reaction (Wofford et al. 1986; Schink 1992) if two-thirds of this ATP unit are invested into translocation of two protons across the cytoplasmic membrane. Two protons could shift the redox potential for two electrons by 165 mV and thus cover exactly the gap between the redox potentials of butyryl-CoA dehydrogenase (–125 mV) and $2\ H^+/H_2$ at 10^{-4} atm (–295 mV).

It is too early to speculate on the mechanism of such a reversed electron transport, specifically whether quinones are involved. One could as well construct a model including only consumption of two protons by hydrogenase activity on the outer face of the cytoplasmic membrane and release of two protons by butyryl-CoA dehydrogenation in the cytoplasm, with transfer of electrons from inside outwards. Elucidation of this system will require further studies, provided that active vesicles can be prepared from this bacterium.

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