Energetics of methanogenic benzoate degradation by *Syntrophus gentianae* in syntrophic coculture

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Growing cocultures of *Syntrophus gentianae* with *Methanospirillum hungatei* degraded benzoate to CH₄ and acetate. During growth, the change of free energy available for *Syntrophus gentianae* ranged between –50 and –55 kJ mol^{–1}. At the end-point of benzoate degradation, a residual concentration of benzoate of 0.2 mM was found, correlating with a free energy change of –45 kJ mol^{–1} available to the fermenting bacterium. Benzoate thresholds were also observed in dense cell suspensions. They corresponded to a final energy situation in the range –31.8 to –45.8 kJ mol^{–1} for the fermenting bacterium. Addition of a H₂-oxidizing sulfate reducer to the methanogenic coculture inhibited by bromoethanesulfonate (BES) resulted in benzoate degradation to below the limit of benzoate detection (10 µM). Accumulated acetate proved to be thermodynamically inhibitory; removal of acetate by *Methanosaeta concilii* in methanogenic or molybdate-inhibited sulfate-reducing cocultures led to degradation of residual benzoate with a final $\Delta G'$ of –45.8 kJ mol^{–1}. In methanogenic cocultures, the residual Gibbs free energy ($\Delta G'$) available for the fermenting bacterium at the end of benzoate degradation correlated with the concentration of acetate built up during the course of benzoate degradation; higher concentrations led to more positive values for $\Delta G'$. Addition of different concentrations of propionate resulted in different values for $\Delta G'$ when benzoate degradation had ceased; higher concentrations led to more positive values for $\Delta G'$. Addition of acetate or propionate to benzoate-degrading cocultures also lowered the rate of benzoate degradation. The protonophore carbonylcyanide chlorophenylhydrazone (CCCP) facilitated further benzoate degradation in methanogenic BES-inhibited cocultures until a $\Delta G'$ of –31 kJ mol^{–1} was reached. We conclude that the minimum energy required for growth and energy conservation of the benzoate-fermenting bacterium *S. gentianae* is approximately –45 kJ (mol benzoate)^{–1}, equivalent to two-thirds of an ATP unit. Both hydrogen and acetate inhibit benzoate degradation thermodynamically, and acetate also partly uncouples substrate degradation from energy conservation.

Keywords: *Syntrophus gentianae*, syntrophic benzoate degradation, benzoate threshold, Gibbs free energy, carbonylcyanide chlorophenylhydrazone (CCCP)

INTRODUCTION

In the absence of external electron acceptors such as

Abbreviations: BES, bromoethanesulfonate; CCCP, carbonylcyanide chlorophenylhydrazone.

oxygen, nitrate or sulfate, benzoate is degraded by fermentative bacteria in syntrophic cooperation with, for example, methanogenic archaea. The fermenting partner produces 3 mol acetate, 3 mol hydrogen and 1 mol CO₂ per mol benzoate degraded (Ferry & Wolfe, 1976; Mountfort & Bryant, 1982; Szewzyk & Schink,

1989): $\text{benzoate}^- + 6\text{H}_2\text{O} \rightarrow 3\text{acetate}^- + 3\text{H}_2 + \text{CO}_2 + 2\text{H}^+$; $\Delta G'^{\circ} = +49.5 \text{ kJ mol}^{-1}$. Acetate and hydrogen both exert a thermodynamic inhibition of benzoate oxidation and are removed in benzoate-degrading cocultures by methanogenic partners (Schink, 1992). In addition to its mass-action effect, acetate may act as an inhibitor of the degradation of benzoate by affecting the proton electrochemical gradient $\Delta\mu_{\text{H}^+}$ and pH homeostasis (Krulwich *et al.*, 1987; Dolfig & Tiedje, 1988; Fukuzaki *et al.*, 1990). In a similar syntrophic cooperation, the methanogenic degradation of butyrate, the partner organisms cooperate in such a manner as to share the available energy in about equal proportions (Schink & Thauer, 1988; Wallrabenstein & Schink, 1994), resulting in about -20 kJ to -25 kJ (mol reaction) $^{-1}$ (corresponding to one-third of an ATP unit; Schink, 1990) for both partners. This assumption was proven experimentally in coculture suspensions (Wallrabenstein & Schink, 1994).

This study focuses on the influence of different pool sizes of acetate and H_2 on benzoate-degrading syntrophic methanogenic associations. Concentrations of the substrate and intermediate and end-products of benzoate degradation were determined in cocultures to yield information on the minimum change of free energy needed by *Syntrophus gentianae* to support energy conservation. Whereas for syntrophic butyrate degradation an equal sharing of energy between the seven reactions involved has been suggested (Schink & Thauer, 1988), so far nothing is known about energy sharing between benzoate-fermenting bacteria and methanogens.

METHODS

Organisms and cultivation. Cocultures of *Syntrophus gentianae* (DSM 8423) with *Methanospirillum hungatei* and pure cultures of *Desulfovibrio desulfuricans* CSN (DSM 9104) were taken from our own culture collection. *Methanosaeta concilii* (DSM 3671) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

The freshwater mineral medium (Widdel & Pfennig, 1981) for cultivation contained 30 mM sodium bicarbonate buffer, 1 mM sodium sulfide as reducing agent, the trace element solution SL 10 (Widdel *et al.*, 1983), selenite-tungstate solution (Tschech & Pfennig, 1984) at a final concentration of 20 nM, and a seven vitamin solution (Widdel & Pfennig, 1984). The pH was adjusted to 7.1–7.3. Cultures were grown under N_2/CO_2 (80:20, v/v) gas phase at 28 °C. Growth was monitored as the change in OD_{578} .

Cell material for physiological experiments was grown in 12 l carboys in a fed-batch manner. Volumes of 4 l were withdrawn every 3–4 d for experiments and were replaced with complete growth medium.

Purity of cultures was checked microscopically during growth in defined mineral medium with benzoate as substrate and after growth tests in complex media containing yeast extract (0.1%), fumarate (5 mM), pyruvate (5 mM) and glucose (5 mM).

Preparation of dense cell suspensions. Cell suspensions of the

coculture of *S. gentianae* and *Msp. hungatei* were harvested in mid-exponential growth phase, transferred to oxygen-free centrifuge vials in an anoxic chamber (Coy) and centrifuged at 8000 g for 30 min. Cells were resuspended in mineral medium without benzoate and centrifuged again. Experiments were performed in butyl-rubber-stoppered 60 ml or 120 ml serum bottles containing cell suspensions (2.5 mg protein ml^{-1}). Pure cultures of *D. desulfuricans* and *Msa. concilii* were grown and harvested the same way.

Benzoate was added from anoxic stock solutions. Samples of 250 μl were withdrawn for benzoate and acetate analysis, and 0.3 ml gas samples for methane and hydrogen analysis at the times indicated.

Chemical analysis. Fatty acids were assayed by GC as described previously (Platen & Schink, 1987). Hydrogen concentrations were determined by GC with a Mercury Reduction Gas Detector (Wolters) after separation at room temperature on a 1.8 m $\times$ 2 mm column packed with 5 Å molecular sieve (Serva). Methane was detected by a flame ionization detector after separation on a molecular sieve column (1 m $\times$ 2 mm) (Matthies & Schink, 1992). Benzoate was quantified by HPLC using a Reversed Phase LiChrosphere 60 RP-select B column (5 μm , 125 $\times$ 4 mm, Merck), an isocratic mobile phase of 50% ammonium phosphate buffer (100 mM, pH 2.6) and 50% methanol, and a detection wavelength of 230 nm (Brune & Schink, 1990). Protein was quantified by a microassay (Bradford, 1976) using bovine serum albumin as standard.

Inhibition experiments. Bromoethanesulfonate (BES) was added from anoxic aqueous stock solutions to 5 mM final concentration, carbonyl cyanide chlorophenylhydrazone (CCCP) from ethanolic stock solutions to a final concentration of 2 nmol (mg protein) $^{-1}$.

Calculations. At the end of each experiment, concentrations of benzoate, acetate and hydrogen were determined. pH values did not change significantly (< 0.2 units) and the H^+ activity was therefore considered to be constant. The change in Gibbs free energy ($\Delta G'$) for benzoate degradation under non-standard conditions was calculated using the measured concentrations of reactants and products according to the following equation:

$$\Delta G' = \Delta G'^{\circ} + RT \ln \frac{[\text{CH}_3\text{COO}^-]^3 [\text{H}_2]^3 [\text{HCO}_3^-]}{[\text{C}_6\text{H}_5\text{COO}^-]}$$

where $[\text{H}_2]$ is the H_2 partial pressure in atmospheres (1 atm = 101325 Pa), R is the gas constant (8.314 J mol^{-1} K $^{-1}$), T is the temperature (all experiments were performed at 301 kelvin) and the values in brackets are the molar concentrations of the respective compounds. In all experiments the bicarbonate concentration equalled 0.03 mol l^{-1} . The change in standard Gibbs free energy ($\Delta G'^{\circ}$) for benzoate degradation ($\text{benzoate}^- + 6\text{H}_2\text{O} \rightarrow 3\text{acetate}^- + 3\text{H}_2 + \text{CO}_2 + 2\text{H}^+$) is $+49.5 \text{ kJ mol}^{-1}$ (Schink, 1992).

RESULTS

Growth experiments

Benzoate was degraded by growing cocultures of *S. gentianae* with *Msp. hungatei*. During degradation, the energetic condition (concentration of substrate and products, temperature, pH) gave a $\Delta G'$ of -55 kJ mol^{-1} to -50 kJ mol^{-1} available to the fermenting bacterium. Degradation ended at a final benzoate concentration of

Fig. 1. Benzoate degradation and acetate formation by a growing coculture of *S. gentianae* and *Msp. hungatei*. ■, Benzoate; ●, acetate; ▲, hydrogen.

0.2 mM, corresponding to a residual $\Delta G'$ of $-44.7 \text{ kJ mol}^{-1}$. Fig. 1 gives a typical example of benzoate degradation and product formation in a growing culture (note differences in scale). All further experiments were carried out with dense cell suspensions to increase reproducibility and conversion rates.

Experiments with dense cell suspensions without manipulation of concentration of products

Dense cell suspensions of syntrophic cocultures of *S. gentianae* with *Msp. hungatei* degraded benzoate at a rate of $6.5 \text{ nmol benzoate min}^{-1} (\text{mg protein})^{-1}$. Benzoate degradation ceased at a final benzoate concentration higher than $10 \mu\text{M}$, even after a prolonged incubation time (10 d), when the residual free energy $\Delta G'$ for *S. gentianae* ranged between -43.2 and $-30.5 \text{ kJ mol}^{-1}$. Table 1(a) gives representative values of final concentrations of substrate and products of benzoate degradation by dense cell suspensions, starting with different initial substrate concentrations. In further

Fig. 2. Effect of addition of *D. desulfuricans* CSN on a benzoate-degrading coculture of *S. gentianae* and *Msp. hungatei* in the presence of 5 mM BES. ■, Benzoate; ●, acetate; ▲, hydrogen. The arrow at $t = 1.3 \text{ h}$ indicates the time of BES addition; the arrow at $t = 4.3 \text{ h}$ indicates addition of strain CSN to the culture.

experiments, the concentrations of the products hydrogen or acetate were manipulated to investigate the reason for the observed benzoate threshold.

Experimental manipulation of the hydrogen partial pressure

After addition of 5 mM BES, an inhibitor of the metabolism of methanogens, to cell suspensions, hydrogen accumulated to values of 100–1000 Pa (Table 1b). Addition of the hydrogen-oxidizing sulfate reducer *D. desulfuricans* CSN to the BES-inhibited culture lowered the H_2 partial pressure to $P_{\text{H}_2} = 0.39 \text{ Pa}$ and initiated further benzoate degradation. At the new equilibrium, no threshold for benzoate was detectable (Fig. 2). This resulted in lower values for $\Delta G'$ (Table 2).

Cocultures of *S. gentianae* with *D. desulfuricans* CSN in the absence of *Msp. hungatei* degraded benzoate to a

Table 1. Final concentrations of benzoate and degradation products reached after benzoate degradation by cell suspensions of *S. gentianae* with *Msp. hungatei*

(a) Without addition of BES; (b) with addition of BES.

Initial acetate (mM)	Initial benzoate (mM)	Final acetate (mM)	Final benzoate (mM)	P_{H_2} (Pa)	Residual $\Delta G'$ (kJ mol^{-1})
(a) 0.3	0.95	3.2	< 0.01	4.8	> -43.2
0.1	2.2	6.3	0.023	9.0	-40.6
0	3.3	9.7	0.01	6.2	-38.2
0.1	3.9	12.2	0.072	11.0	-36.9
0	5.17	15.3	0.028	8.7	-34.6
0.1	5.85	17.8	0.012	6.9	-33.1
0	8.3	21.9	0.937	7.3	-30.5
(b) 0.3	14.2	3.75	12.9	95	-42.6
0.1	17.6	2.5	16.8	140	-43.3
0	19.0	1.1	18.6	290	-44.3
0.1	24.3	0.25	24.2	1300	-44.8

Table 2. Final concentrations of benzoate and degradation products in benzoate-degrading cell suspensions of *S. gentianae* with *Msp. hungatei* inhibited by BES after addition of *D. desulfuricans* CSN

Initial acetate (mM)	Initial benzoate (mM)	Final acetate (mM)	Final benzoate (mM)	P_{H_2} (Pa)	Residual $\Delta G'$ (kJ mol ⁻¹)
0	1.57	4.7	< 0.01	0.34	> -65.2
0	2.09	6.3	< 0.01	0.52	> -59.8
0.1	4.1	12.2	< 0.01	0.26	> -60.1
0.1	6.3	18.7	< 0.01	0.42	> -53.3

Fig. 3. Effect of acetate removal by *Msa. concilii* on a benzoate-degrading coculture of *S. gentianae* and *Msp. hungatei* that showed a detectable benzoate threshold. ■, Benzoate; ●, acetate. The arrow indicates the time of addition of *Msa. concilii*. **Fig. 4.** Effect of addition of a dense cell suspension of *Msa. concilii* to a molybdate-inhibited benzoate-degrading coculture of *S. gentianae* and *D. desulfuricans* CSN. ■, Benzoate; ●, acetate; ▲, hydrogen. The arrow indicates the time of addition of *Msa. concilii*.

remnant concentration below the detection limit (10 μ M; data not shown). Inhibition of the sulfate reducer during the degradation process by addition of 5 mM sodium molybdate resulted in increased hydrogen partial pressures (up to 750 Pa) and no further benzoate degradation. Residual $\Delta G'$ values ranged between -45.0 kJ mol⁻¹ and -42.5 kJ mol⁻¹.

Fig. 5. Effect of increasing final concentrations of acetate on the $\Delta G'$ in cocultures of *S. gentianae* and *Msp. hungatei* (●) or *S. gentianae* and *D. desulfuricans* CSN (○) additions at different concentrations on the $\Delta G'$ at the time of no further benzoate degradation by the coculture of *S. gentianae* and *Msp. hungatei*.

Experimental manipulation of the acetate concentration

As both hydrogen and acetate are produced at a ratio of three mols per mol benzoate oxidized, a thermodynamic inhibition by acetate should be as likely as inhibition by hydrogen. Removal of acetate by addition of the aceticlastic methanogen *Methanosaeta concilii* to methanogenic cocultures led to further degradation of benzoate (Fig. 3). The same effect was observed after addition of *Methanosaeta concilii* to sulfate-reducing cocultures that were inhibited by molybdate: benzoate was degraded further until a residual $\Delta G'$ of -45.8 kJ mol⁻¹ in the system was reached (Fig. 4). Acetate additions to sulfate-reducing cocultures to concentrations of 20, 40, 60 or 80 mM did not result in detectable benzoate threshold concentrations (data not shown).

Correlation between acetate concentrations and $\Delta G'$

The results described above showed that the $\Delta G'$ value available for the benzoate degrader varies with the final acetate concentrations in methanogenic or sulfate-reducing cocultures. There was a positive correlation between the final acetate concentration and the residual Gibbs free energy observed. Increasing acetate concentrations correlated with higher residual $\Delta G'$ values (Fig. 5). The correlation between final $\Delta G'$ and the con-

Fig. 6. Effect of addition of 2 nmol CCCP (mg protein)⁻¹ to a BES-inhibited benzoate-degrading coculture of *S. gentianae* and *Msp. hungatei*. ■, Benzoate; ●, acetate; ▲, hydrogen. The arrow at $t = 1.3$ h indicates the time of addition of BES; the arrow at $t = 2.3$ h indicates the time of addition of CCCP.

centration of acetate when no further benzoate was degraded in cell suspensions without BES and in cell suspensions amended with 5 mM BES were similar. The same correlation was observed with sulfate-reducing cocultures that were inhibited by molybdate (data not shown).

Externally added sodium acetate (50 mM) inhibited benzoate degradation, and sodium propionate additions (50 mM) proved to be inhibitory as well. The additions also decreased degradation rates: the rate of benzoate degradation decreased from 6.7 nmol benzoate min⁻¹ (mg protein)⁻¹ to 3.1 and 0.83 nmol benzoate min⁻¹ (mg protein)⁻¹ with 50 mM propionate and 50 mM acetate, respectively. Addition of propionate (50 mM) to benzoate-degrading cultures showed a similar effect on $\Delta G'$ at the end-point of degradation, but not as much as with acetate (Fig. 5). Sodium chloride additions (50 mM) had no effect.

Addition of 2 nmol CCCP (mg protein)⁻¹ to a BES-inhibited methanogenic coculture resulted in further degradation of benzoate. The $\Delta G'$ at the end-point of this reaction was about -31 kJ mol⁻¹ (Fig. 6). Control assays with ethanol alone did not show any effect.

DISCUSSION

In this study, benzoate threshold concentrations during methanogenic benzoate degradation by growing cultures or dense cell suspensions are reported for the first time. In our experiments with *S. gentianae* and *Msp. hungatei* as partner organisms, benzoate threshold values higher than 10 μ M could be detected. Warikoo *et al.* (1996) found benzoate thresholds higher than 0.2 μ M in a sulfate-reducing coculture only if exogenous acetate was added. Thermodynamic limitations are likely to cause the threshold concentrations in methanogenic cocultures for the following reasons. (i) Hydrogen accumulated in BES-inhibited methanogenic cocultures to partial pressures about 12.5 times higher than in non-inhibited cocultures concurrent with higher threshold values for benzoate than in non-inhibited cocultures. (ii) If hydrogen was removed from the headspace of such

cocultures by flushing with N₂/CO₂, benzoate degradation resumed with further hydrogen production until the same partial pressure was reached as before. (iii) Addition of *D. desulfuricans* to a BES-inhibited methanogenic coculture exhibited a further decrease in benzoate concentration as the hydrogen partial pressure was lowered.

A thermodynamic limitation of benzoate degradation by hydrogen could also be shown in sulfate-reducing cocultures where increasing hydrogen partial pressure (due to inhibition of the sulfate reducer) led to the inhibition of benzoate degradation and to observable benzoate thresholds. These results clearly show that benzoate degradation is thermodynamically limited by the hydrogen partial pressure that builds up in the culture.

As both hydrogen and acetate are produced at stoichiometric ratios of 3 mol per mol benzoate oxidized, thermodynamic inhibition by acetate should be as likely as inhibition by hydrogen. This assumption could be proved by showing that benzoate degradation resumed after removal of acetate from methanogenic cell suspensions that had ceased degrading benzoate. A comparable effect was seen in butyrate-degrading associations (Ahling & Westermann, 1988). The same effect was observed with sulfate-reducing cocultures. Acetate was removed from molybdate-inhibited cocultures that had reached a condition that did not allow any further benzoate degradation, and removal led to further degradation of benzoate, showing that acetate is a product that influences the degradation thermodynamically.

After benzoate degradation ceased, the residual $\Delta G'$ available to the fermenting organism depended on the concentration of acetate that had built up in the course of benzoate degradation. An additional effect of increasing concentrations of acetate in the growth medium may arise because the undissociated acid might act as an uncoupler of the Δ pH component of the proton electrochemical gradient (Herrero *et al.*, 1985; Krulwich *et al.*, 1987; Fukuzaki *et al.*, 1990). Menzel & Gottschalk (1985) measured the intracellular pH in growing cultures of *Acetobacterium wieringae* and *Acetobacter aceti* and found a decrease in intracellular pH due to an increase in extracellular acetic acid. They demonstrated that neither the anaerobic nor the aerobic acetogen was able to maintain a large Δ pH in the presence of high concentrations of acetic acid. It is surprising that in our experiments acetate exhibited an uncoupling effect at comparably low concentrations. However, since the substrate turnover rate and the energy supply of the benzoate-fermenting cells is comparably low, minor uncoupling effects may also become measurable.

Addition of the protonophore CCCP to dense cell suspensions of *S. gentianae* inhibited by BES resulted in further benzoate degradation. This finding suggests that benzoate degradation is linked to the generation of a proton motive force. Lowering the electrochemical gradient $\Delta\mu_{H^+}$ would be expected to stimulate benzoate

degradation and increase the hydrogen partial pressure. If the remaining force to drive this process is insufficient ($> -45 \text{ kJ mol}^{-1}$) no further protons can be translocated and benzoate degradation ceases. Lowering the $\Delta\mu_{\text{H}^+}$ by addition of CCCP allows further proton translocation, and benzoate degradation and hydrogen production resume. However, we found that the reaction did not run to its equilibrium at $\Delta G' = 0$ upon addition of CCCP, perhaps because ATP is necessary for initial activation of benzoate and ATP might be partly hydrolysed by ATPase in the presence of a decreased proton gradient.

The correlation given between $\Delta G'$ and the concentration of acetate in the system suggests that acetate depolarizes the membrane in a manner similar to the protonophore CCCP. Additional evidence for this assumption is given by the fact that both the effect of CCCP and the proposed uncoupling effect of acetate lead to further degradation of benzoate which ceases when there is -30 kJ mol^{-1} available to the fermenting bacterium. To detect the limit of the $\Delta G'$ that is necessary to run the benzoate degrading pathway with conservation of energy, it was necessary to exclude the postulated uncoupling effects of acetate. Acetate depletion by *Msa. concilii* gave a limit in $\Delta G'$ of $-45.8 \text{ kJ mol}^{-1}$ at low acetate concentrations. This amount of energy is obviously the minimum amount necessary for energy conservation by the benzoate-fermenting bacterium *S. gentianae*. The same value was also reported as the degradation end-point energy in sulfate-reducing cocultures of the syntrophic benzoate-degrader strain SB and *Desulfovibrio* sp. strain G-11 (Warikoo *et al.*, 1996).

Calculated values for the $\Delta G'$ available to the fermenting bacterium in growing cultures as well as in dense cell suspensions while benzoate was degraded were about twice as high as values calculated for bacteria oxidizing butyrate in methanogenic environments (Schink & Thauer, 1988). It equals two-thirds of an ATP unit and is therefore twice as much as needed by butyrate-fermenting syntrophic bacteria for their energy conservation (-23 kJ mol^{-1} ; Wallrabenstein & Schink, 1994). Future work will have to elucidate how *S. gentianae* can balance its overall energy budget with $-45 \text{ kJ (mol benzoate degraded)}^{-1}$.

In natural or seminatural habitats such as sediments or sewage sludge digesters, benzoate is degraded to methane and CO_2 by a community of three metabolically different types of bacteria cooperating in 19 partial reactions per four mols benzoate degraded (Schink, 1992); $4\text{C}_6\text{H}_5\text{COO}^- + 4\text{H}^+ \rightarrow 15\text{CH}_4 + 13\text{CO}_2$; $\Delta G'^{\circ} = -624 \text{ kJ per 4 mol benzoate}$. Based on the results presented in this paper, it has to be assumed that the first reaction running four times needs a $\Delta G'$ of -45 kJ mol^{-1} . Taking this into consideration, an amount of $-29.5 \text{ kJ mol}^{-1}$ remains for each of the further 15 reactions. Even a benzoate concentration as low as 10^{-8} M would still yield $-45 \text{ kJ per reaction}$ catalysed by the fermenting bacterium and $< -20 \text{ kJ}$ for

every partial reaction catalysed by the methanogenic partners. This amount of energy represents the lower limit required for ATP formation in bacteria (Schink, 1990). Thus, benzoate degradation by methanogenic microbial communities would not be limited thermodynamically at benzoate concentrations down to 10^{-8} M . So far, no free benzoate concentrations in natural or semi-natural environments have been reported.

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