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Ludger Schöcke · Bernhard Schink

Energetics and biochemistry of fermentative benzoate degradation by *Syntrophus gentianae*

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Abstract The pathway of fermentative benzoate degradation by the syntrophically fermenting bacterium *Syntrophus gentianae* was studied by measurement of enzyme activities in cell-free extracts. Benzoate was activated by a benzoate-CoA ligase reaction, forming AMP and pyrophosphate, which was subsequently cleaved by a membrane-bound proton-translocating pyrophosphatase. Glutaconyl-CoA (formed from hypothetical pimelyl-CoA and glutaryl-CoA intermediates) was decarboxylated to crotonyl-CoA by a sodium-ion-dependent membrane-bound glutaconyl-CoA decarboxylase, a biotin enzyme that could be inhibited by avidin. The overall energy budget of this fermentation could be balanced only if the dearomatizing reduction of benzoyl-CoA is assumed to produce cyclohexene carboxyl-CoA rather than cyclohexadiene carboxyl-CoA, although experimental evidence of this reaction is still insufficient. With this assumption, benzoate degradation by *S. gentianae* can be balanced to yield one-third to two-thirds of an ATP unit per benzoate degraded, in accordance with earlier measurements of whole-cell energetics.

Key words *Syntrophus gentianae* / Syntrophic benzoate degradation / Glutaconyl-CoA decarboxylase · Proton-translocating pyrophosphatase · Benzoyl-CoA reductase (dearomatizing)

Introduction

Bacteria active in the terminal steps of degradation of organic matter to methane and carbon dioxide have to operate under conditions of extremely low energy supply in the range of only fractions of one ATP unit per substrate molecule degraded (Schink 1991, 1997; Stams 1994). Benzoate is one of the products of primary fermentation of organic matter and is formed as an intermediate, e.g., during degradation of aromatic amino acids. Assessments of the energetics of benzoate degradation by syntrophically fermenting bacteria such as *Syntrophus buswellii* or *Syntrophus gentianae* on the basis of substrate and product measurements in suspensions of intact cells with and without partner bacteria have shown that these bacteria have an energy span of approximately –40 to –45 kJ available per mol benzoate degraded, equivalent to one-third to two-thirds of an ATP unit (Warikoo et al. 1996; Schöcke and Schink 1997).

The biochemistry of anaerobic benzoate degradation has been studied mainly with nitrate-reducing bacteria and with phototrophic bacteria. In both cases, benzoate is activated initially by an ATP-dependent benzoate-CoA ligase, forming AMP and pyrophosphate, which is further hydrolyzed to inorganic phosphate (Geissler et al. 1988; Fuchs et al. 1994), thus consuming two ATP hydrolysis equivalents in the activation reaction. The nitrate reducer *Thauera aromatica* reduces benzoyl-CoA to cyclohex-1,5-diene carboxyl-CoA (Koch and Fuchs 1992; Koch et al. 1993) probably in two one-electron reduction steps, both of which require stoichiometric hydrolysis of one ATP per reaction (Boll and Fuchs 1995), adding up to four ATP units hydrolyzed before the aromatic ring is cleaved. Nitrate-reducing bacteria can recover these ATP investments through β-oxidation of the aliphatic C-7 dicarboxylic acid residue derived upon hydrolytic ring opening and oxidation of the formed acetyl-CoA residues. Syntrophically benzoate-degrading bacteria excrete the acetyl residues as acetate and can obtain only one ATP unit per acetyl residue through phosphotransacetylase and acetate kinase reactions, leaving a gap of at least one ATP unit per benzoate oxidized if these bacteria used the same reaction steps as the nitrate-reducing bacteria do.

This obvious contradiction in the overall energy balance of these bacteria – which had also been discussed earlier (Boll and Fuchs 1995) – caused us to elucidate the reaction steps involved in benzoate degradation by the

Ludger Schöcke · Bernhard Schink (✉)
Fakultät für Biologie, Mikrobielle Ökologie,
Universität Konstanz, Postfach 5560,
D-78434 Konstanz, Germany
e-mail: bernhard.schink@uni-konstanz.de
Tel. +49-7531-882140; Fax +49-7531-882966

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syntrophically benzoate-degrading fermenting bacterium *S. gentianae*.

Materials and methods

Organisms and cultivation

Cocultures of *S. gentianae* (DSM 8423) with *Methanospirillum hungatei* were taken from our own culture collection. The freshwater mineral medium (Widdel and 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 and Pfennig 1984) at a final concentration of 20 nM, and a seven-vitamin solution (Widdel and 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 change in OD_{578}.

Cell material for biochemical experiments was grown in 12 l carboys in a fed-batch manner. Volumes of 4 l were withdrawn every 3–4 days for experiments and were replaced with complete growth medium. The purity of the 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%, w/v), fumarate (5 mM), pyruvate (5 mM), and glucose (5 mM).

Preparation of cell-free extracts

Cell suspensions of the coculture of *S. gentianae* with *M. hungatei* were harvested in the mid-exponential growth phase, transferred into oxygen-free centrifuge vials in an anoxic chamber (Coy, Ann Arbor, Mich., USA), and centrifuged at 8,000 × *g* for 30 min. Cells were resuspended in mineral medium without benzoate and centrifuged again. For selective cell lysis, the pellet was resuspended in Tris-HCl buffer (10 mM, pH 8.0) containing 0.5 mg lysozyme ml^{-1} and 10 mM EDTA. The final concentration of lysozyme was 2.5–5 μg (mg cell protein)$^{-1}$. Cells were incubated for 30–45 min at 37 °C, and lysis was followed microscopically.

For localization of enzymes in cell membranes or the cytoplasm, the buffer contained in addition 20% sucrose (w/v). Spheroplasts were centrifuged gently (20 min at 5,000 × *g*) and were stabilized by the addition of $MgCl_2$ to a final concentration of 11 mM.

Fractionation of cell-free extracts

A volume of 3 ml of a sucrose solution (20%, w/v) was overlayed by 0.5 ml cell-free extract containing less than 10 mg protein ml^{-1} and was centrifuged for 90 min at 100,000 × *g* and 4 °C in an Optima TL ultracentrifuge (Beckman). The supernatant (cytosol) was carefully removed with a pasteur pipette in an anoxic chamber. The pellet containing the membrane fraction was resuspended in Tris-HCl buffer (pH 8.0), washed once in the same buffer, centrifuged again under the same conditions, and resuspended in the same buffer.

Determination of enzyme activities

Enzyme activities were measured either continuously by spectroscopy or discontinuously by quantification of products and/or substrates. All activities were measured in at least three independent parallel assays at room temperature except where stated otherwise. Care was taken that the enzyme activity was proportional to the protein content applied in the experiment. Control experiments with heat-denatured extracts were run to verify that the activities found were enzyme-catalyzed.

Aromatic-acid-CoA ligase (EC 6.2.1.-) was measured discontinuously. Cell-free extracts were added to 20 mM triethanolamine-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 1 mM ATP, 1 mM CoASH, and 1 mM of the respective substrate (benzoate, 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, or 2,5-dihydroxybenzoate). Alternatively, the ligase was measured by coupling the reaction via ATP consumption to pyruvate kinase (EC 2.7.1.40), myokinase (EC 2.7.4.3), and lactate dehydrogenase (EC 1.1.1.27) (Geissler et al. 1988).

Acyl-CoA:acceptor transferase (EC 2.8.3.-) was measured in a manner analogous to aromatic-acid-CoA ligase (discontinuous) except that acetyl-CoA, acetoacetyl-CoA, or benzoyl-CoA was added instead of ATP plus CoASH.

Glutaryl-CoA dehydrogenase (EC 1.3.99.7) was measured by a method modified according to Stams et al. (1984): in 50 mM potassium phosphate buffer (pH 7.2), 0.1% (w/v) Triton X-100, 1 mM $K_3[Fe(CN)_6]$, and 0.1 mM phenazine methosulfate, the reaction was started with 0.5 mM glutaryl-CoA. Reduction of $K_3[Fe(CN)_6]$ was followed at a wavelength of 420 nm (ε_{420} = 0.9 mM^{-1} cm^{-1}).

Glutaconyl-CoA decarboxylase (EC 4.1.1.70) was measured in a coupled assay containing 50 mM potassium phosphate buffer (pH 7.2), 0.1% (w/v) Triton X-100, 20 mM NaCl, 2 mM dithioerythritol, 2 mM EDTA, 1 mM NAD^+, 1 mM acetylphosphate, 0.0125 mM CoASH, 1 mM Na glutaconate, and a set of five coupling enzymes prepared from *Acidaminococcus fermentans* (Buckel 1986). Reduction of NAD^+ was followed at 334 nm (Gorny and Schink 1993). Dependence of the activity on Na^+ was determined in buffer that was prepared from almost sodium-free components (< 50 μg Na^+ g^{-1}) and was run over a cation-exchange column (HiTrap SP, 1 ml; Pharmacia) pre-equilibrated with 3 M KCl prior to enzyme measurements. Glutaconate was supplied as a KOH-neutralized glutaconic acid stock solution. All solutions were kept in quartz or plastic containers rinsed with Millipore-purified water.

Avidin as a specific inhibitor of biotin enzyme reactions was added from stock solutions to a final concentration of 0.4 mg ml^{-1}. Cell-free extracts were preincubated with avidin for 15 min before enzyme assay.

3-Hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) was measured via oxidation of NADH at 365 nm. The assay mixture contained 0.1 mM NADH and 0.3 mM acetoacetyl-CoA in 100 mM potassium phosphate buffer (pH 7.2) (modified according to Bergmeyer 1983).

β-Ketothiolase (EC 2.3.1.16) was measured spectrophotometrically (Lynen and Ochoa 1953) following decrease in acetoacetyl-CoA concentration at 303 nm. Acetate kinase (EC 2.7.2.1) and phosphotransacetylase (EC 2.3.1.8) were measured according to standard methods (Bergmeyer 1983).

Hydrogenase (EC 1.18.99.1) and formate dehydrogenase (EC1.2.1.2) were measured via benzyl viologen reduction (Diekert and Thauer 1978).

Benzoyl-CoA reductase was measured according to Koch and Fuchs (1992).

Chemical analyses

Protein concentrations were determined by a commercially available kit (Pierce) with bovine serum albumin as the standard. ATP, ADP, and AMP were separated and measured via ion pair chromatography (Litters and Schmelzeisen-Redeker 1989) on an Ultrasphere ODS column (5-μm diameter, 4.6 × 150 mm; Beckman) with 80 mM KH_2PO_4 (adjusted with 1 M KOH to pH 6.0), 5 mM tetrabutylammonium hydrogensulfate in methanol (77 : 23, v/v) as eluent at a flow rate of 1.25 ml min^{-1}. Detection was at 260 nm.

Chemicals

All chemicals were of analytical or reagent grade and were obtained from Biomol (Ilvesheim, Germany), Boehringer (Mannheim, Germany), Eastman Kodak (Rochester, N.Y., USA), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Pharmacia (Freiburg, Germany), Serva (Heidelberg, Germany), and Sigma (Deisenhofen, Germany). Gases were purchased from Messer-

Griesheim (Darmstadt, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Results

Measurement of enzymatic activities in cell-free extracts

Activities of enzymes involved in energy metabolism during growth of *S. gentianae* with benzoate were determined in cell-free extracts of cells that were opened selectively by lysozyme treatment, leaving the methanogenic partner cells intact (Table 1). Acetate kinase and phosphotransacetylase exhibited low but reproducible activities. Acetate kinase could be detected by the test system applied only after removal of the cell membranes and in the presence of the ATPase inhibitor dicyclohexyl carbodiimide. Hydrogenase activity was far higher than formate dehydrogenase activity, indicating that interspecies electron transfer was largely through hydrogen rather than through formate. There was no benzoate : acyl-CoA transferase activity either with acetyl-CoA or with acetoacetyl-CoA as CoA donors, or with gentisate or benzoate as acceptors. Instead, we found good activity of a benzoate-CoA ligase that formed AMP and PP_i as coproducts. No reversible, ADP-forming acetyl-CoA synthetase activity could be detected. There was a high activity of pyrophosphatase; this activity was shown to be membrane-bound and has been characterized in a separate paper (Schöcke and Schink 1998). Of the enzymes involved in further degradation of the aliphatic dicarboxylic acid derivative formed upon ring cleavage, glutaryl-CoA dehydrogenase and glutaconyl-CoA decarboxylase were found at reasonable activities. Some of these enzymes were characterized in more detail.

Table 1 Enzyme activities measured in cell-free extracts of *Syntrophus gentianae* grown in coculture with *Methanospirillum hungatei*. The substrate turnover rate of the growing culture was 80–100 nmol min^{-1} (mg protein)$^{-1}$. Standard deviations (n = 5) are indicated (*nd* not detectable)

Enzyme	Specific activity[a] [nmol min^{-1} (mg protein)$^{-1}$]
Acetate kinase	20 ± 4
Propionate kinase	< 1
Phosphotransacetylase	57 ± 7
Hydrogenase[a]	1,900 ± 90
Formate dehydrogenase[a]	66 ± 4
Benzoyl-CoA transferase[b]	nd
Benzoate-CoA ligase	210 ± 5
Glutaryl-CoA dehydrogenase	155 ± 12
Glutaconyl-CoA decarboxylase	48 ± 4
Pyrophosphatase	1,210 ± 70
β-Ketothiolase	5,300 ± 67
3-Hydroxybutyryl-CoA dehydrogenase	5,374 ± 62

[a] Measured with benzyl viologen as electron donor
[b] With either acetate or acetoacetate as acceptor

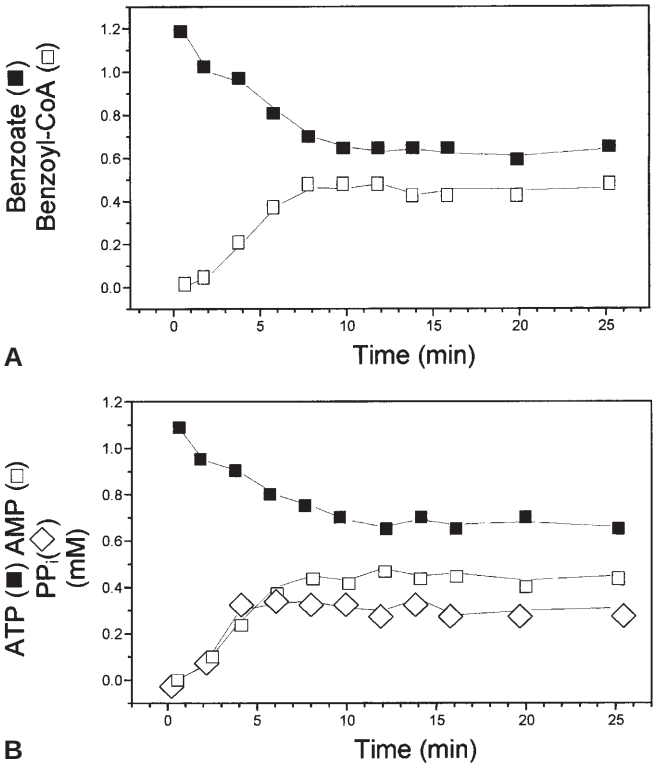

Fig. 1 A, B Benzoate-CoA ligase reaction in cell-free extracts of *Syntrophus gentianae*. **A** Decrease of benzoate (■) and accumulation of benzoyl-CoA (□). **B** Conversion of ATP (■) to AMP (□) and pyrophosphate (◇)

Table 2 Specific aromic acid-CoA ligase activities in cell free extracts of *Syntrophus gentianae*. Standard deviations (n = 5) are indicated

Aromic acid-CoA ligase activity with	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]
Benzoate	210 ± 5
2-Hydroxybenzoate	15 ± 2
3-Hydroxybenzoate	22 ± 1
4-Hydroxybenzoate	< 0.2
2,5-Dihydroxybenzoate	174 ± 15

Aromatic-acid CoA ligase activity

Benzoate-CoA ligase was found at high activity. Per mol benzoyl-CoA formed, 1 mol of ATP was transformed to AMP and PP_i (Fig. 1). The reaction was linear with time for approximately 5–6 min. Besides benzoate, also gentisate, salicylate, and 3-hydroxybenzoate were activated by this reaction system. Salicylate and 3-hydroxybenzoate were activated at rates approximately one-tenth of that of the other aromatic substrates (Table 2). Affinity constants K_m for benzoate, ATP, and CoASH were 0.026, 0.05, and 0.8 mM, respectively.

Table 3 Effect of Triton X-100, avidin, and biotin on glutaconyl-CoA decarboxylase activity in the membrane fraction. Avidin was added to the assay at a concentration of 0.4 mg ml^{-1} before cell-free extract was added. In the last experiment, avidin was preabsorbed with 0.8 mM biotin

Addition	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]	
	No addition	With 0.1% Triton X-100
None	22.5	17
Avidin	1.8	1.0
Avidin + biotin	21.3	17.7

Glutaconyl-CoA decarboxylase activity

Glutaconyl-CoA decarboxylase activity was measured by coupling its activity to those of five auxiliary enzymes that supply the substrate glutaconyl-CoA and couple the reaction to the reduction of NAD^+ at a rate equivalent to that of the decarboxylation. The specific activity of the enzyme in crude extracts was 22.5 nmol min^{-1} (mg protein)$^{-1}$ in the presence of 20 mM NaCl in the assay mixture.

After separation of the cytosolic fraction and the membrane fraction, the activity was detected almost exclusively (96.3%) in the membranes. The cytosolic enzyme 3-hydroxybutyryl-CoA dehydrogenase was found exclusively (> 97.5%) in the cytosolic fraction, indicating that the membrane fraction was not significantly contaminated with enzymes of the cytosolic fraction. When 1% Triton X-100 was applied to cell-free extracts, the decarboxylase activity was found in the cytosolic fraction (89.4%), indicating that the enzyme was solubilized by this treatment. Avidin (0.4 mg ml^{-1}) inhibited the decarboxylation activity; addition of biotin reactivated the enzyme partially. Avidin preincubated with excess biotin did not inhibit the activity (Table 3). These results suggest that the decarboxylase is a biotin enzyme. Addition of 0.1% Triton X-100 (approximately 1 mg Triton X-100 per mg protein) did not result in solubilization of the enzyme but decreased the measurable activities slightly.

The decarboxylase activity depended strictly on the presence of sodium ions (Fig. 2). In comparison to an essentially sodium-free (< 20 μM) control, 20 mM NaCl increased the activity 220-fold. In assays with low Na^+ ion concentrations, KCl was added to keep the ionic strength constant in all experiments. Double reciprocal plots (not shown) gave a K_m value for Na^+ of 2.5 mM and a V_{max} of 22.5 nmol min^{-1} (mg protein)$^{-1}$. Dependence of the glutaconyl-CoA decarboxylase activity on sodium ions and its localization in the cytoplasmic membrane strongly indicate that this enzyme acts as a primary sodium-ion pump that establishes a sodium ion gradient across the cytoplasmic membrane.

Benzoyl CoA reductase

Attempts to apply a continuous in vitro enzyme assay (Koch and Fuchs 1992) for benzoyl-CoA reductase (dearomatizing) activity in cell-free extracts did not succeed although controls with cell-free extracts of *T. aromatica* were positive. Variation of the concentration of reactants or cell-free extracts, or variation of the electron donor [dithionite, Ti(III) citrate, or ferredoxin from *Clostridium pasteurianum*) also did not result in any measurable activity.

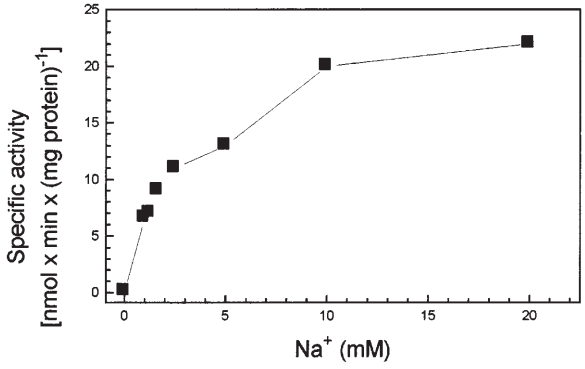

Fig. 2 Dependence of membrane-bound glutaconyl-CoA decarboxylase of *Syntrophus gentianae* on the Na^+ ion concentration. The background Na^+ ion concentration was 17 μM

Discussion

In the present study, the pathway of benzoate degradation by a syntrophically fermenting bacterium was studied on the basis of enzyme measurements in cell-free extracts. The studies revealed that the degradation pathway differs from that of the denitrifying bacterium *T. aromatica* in some key reactions. The hydrolysis of pyrophosphate (Schöcke and Schink 1998) and the decarboxylation of glutaconyl-CoA were shown to be membrane-bound enzyme activities that conserve part of the free-energy change of the respective reaction by translocation of ions across the membrane. The substrate activation reaction, on the other hand, was the same as in the nitrate-reducing bacterium.

Activation of benzoate to benzoyl-CoA in *S. gentianae* was not catalyzed by CoA exchange with other activated acyl residues, e.g., acetyl-CoA or acetoacetyl-CoA. Such transferase activities are commonly used for substrate activation among fermenting bacteria because they allow substrate activation with a minimum expense of energy, equivalent to one ATP unit. Nevertheless, the syntrophically fermenting bacterium *S. gentianae* uses not a transferase but a benzoate-CoA ligase for benzoate activation, requiring ATP as cosubstrate and forming AMP plus pyrophosphate as coproducts. The equilibrium of this reaction is on the side of the substrates, analogous to the reaction:

$$\text{Acetate} + \text{CoASH} + \text{ATP} \rightarrow \text{Acetyl-CoA} + \text{AMP} + PP_i$$

$\Delta G^{\circ\prime} = +3$ kJ mol^{-1} (Thauer et al. 1977)

and the equilibrium is shifted in most known cases to the side of the products by subsequent hydrolysis of pyrophosphate:

$$\text{Benzoate} + \text{CoASH} + \text{ATP} \rightarrow \text{Benzoyl CoA} + \text{AMP} + 2\ P_i$$

$\Delta G^{\circ\prime} = -28$ kJ mol^{-1} (Thauer et al. 1977)

This equilibrium shift is accomplished with a substantial amount of energy that is usually lost for the bacterium's metabolism. *S. gentianae*, on the other hand, conserves part of this energy by a membrane-bound pyrophosphatase reaction that couples pyrophosphate hydrolysis with the translocation of at least one proton across the cytoplasmic membrane (Schöcke and Schink 1998). If the energy amount for this translocation is calculated to be equivalent to one-third of an ATP unit under non-equilibrium conditions (20–22 kJ per proton transported; Schink 1997), the overall reaction reads as follows:

$$\text{Benzoate} + \text{CoASH} + \text{ATP} + H^+_{in} \rightarrow \text{Benzoyl CoA} + \text{AMP} + 2\ P_i + H^+_{out}$$

$\Delta G^{\circ\prime} = -6$ kJ mol^{-1}

Thus, coupling of these three reactions combines a favorable shift of the reaction equilibrium with a minimum expenditure of metabolic energy.

A further energy-conserving step was found in the decarboxylation of the C5-dicarboxylic acid residue glutaconyl-CoA, which is formed during β-oxidation of the aliphatic residue formed upon ring hydrolysis. Unlike the nitrate-reducing bacteria *T. aromatica* and *Azoarcus sp*. (formerly reported as *Pseudomonas* strains K172 and KB740; Härtel et al. 1993), which catalyze the decarboxylation simultaneously with the glutaryl-CoA dehydrogenase reaction in the cytoplasm without energy conservation, *S. gentianae* employs a membrane-bound enzyme that is strictly sodium-dependent and likely to couple the decarboxylation with a translocation of sodium ions across the membrane. Such enzymes have been studied in detail with *Acidaminococcus fermentans* (Buckel and Semmler 1983; Buckel 1986; Bendrat and Buckel 1993), and a similar enzyme system even acts as the sole energy source for a bacterium running its entire energy metabolism on the basis of glutarate decarboxylation to butyrate and isobutyrate (Matthies and Schink 1992). The sodium ions transported across the cytoplasmic membrane may give rise to ATP formation either directly by a membrane-bound sodium-ion-pumping ATPase (Dimroth 1997) or by a proton-pumping ATPase after translation into a proton gradient through a proton/Na^+ antiporter. Alternatively, the established sodium-ion gradient could play a role in substrate uptake and thus save energy stored in proton gradients.

The dearomatizing reduction of benzoyl-CoA in *T. aromatica* appears to proceed in two single electron transfer steps, each of which is coupled to the hydrolysis of one ATP. In the first step, one electron could be added to form the benzoyl-CoA radical anion, which is stabilized by uptake of a proton. Hereafter, a second electron is introduced, and upon protonation the cyclohexadiene derivative is formed as a first stable reaction product (Buckel and Keese 1995). This initially hypothetical reaction mechanism has received experimental support from EPR

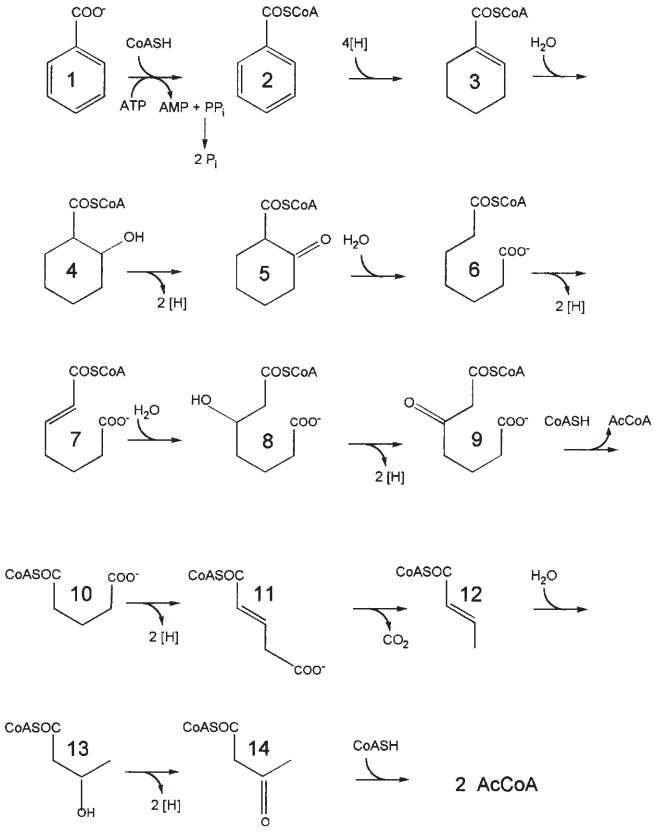

Fig. 3 Pathway of benzoate degradation by *Syntrophus gentianae* suggested on the basis of the results of the present study. *Numbers* refer to the following compounds: *1* benzoate, *2* benzoyl-CoA, *3* cyclohex-1-ene carboxyl-CoA, *4* 2-hydroxycyclohexane carboxyl-CoA, *5* 2-oxocyclohexane carboxyl-CoA, *6* pimelyl-CoA, *7* dehydropimelyl-CoA, *8* 3-hydroxypimelyl-CoA, *9* 3-oxopimelyl-CoA, *10* glutaryl-CoA, *11* glutaconyl-CoA, *12* crotonyl-CoA, *13* 3-hydroxybutyryl-CoA, and *14* acetoacetyl-CoA

investigations (Boll et al. 1997). Thus, two ATP are hydrolyzed in the overall process (Boll and Fuchs 1995; Heider and Fuchs 1997). This ATP expenditure is necessary because the standard redox potential of the couple benzene/cyclohexadiene is very low [–620 mV; calculated according to free-energy values published by D'Ans and Lax (1983)] and because reduction of benzene to cyclohexadiene, even with electrons at the hydrogen level, requires an energy input in the range of at least 40 kJ per mol. The energetics of the corresponding reactions with the carboxyl-CoA derivatives should not be substantially different. As outlined above, the syntrophically fermenting bacteria cannot afford to spend this amount of energy in the reduction reaction. Based on preliminary experiments with radiolabeled [ring-^{14}C] benzoate (results not shown), we assume that *S. gentianae* forms a more reduced product from benzoyl-CoA than does *T. aromatica*, e.g., a cyclohexene rather than a cyclohexadiene derivative in the dearomatizing reaction. The redox potential of this reduction reaction (benzene/cyclohexene) is at –350 mV and could therefore be accomplished with electrons at the potential of the NAD^+/NADH couple (–320 mV) with-

out further energy investment. Future work will have to deal with the chemistry of this reduction reaction, which can only be hypothesized at present.

The pathway of benzoate degradation by *S. gentianae* as suggested on the basis of the results of the present study, results of previous studies with denitrifiers and phototrophs (Heider and Fuchs 1997) and the assumptions above is depicted in Fig. 3. Three ATP are synthesized in the phosphotransacetylase and acetate kinase reactions, converting three acetyl-CoA to free acetate. Hydrolysis of pyrophosphate by the membrane-bound pyrophosphatase is coupled to proton translocation, equivalent to one-third of an ATP, and a further one-third of an ATP unit is formed via sodium-ion translocation by the glutaconyl-CoA decarboxylase reaction (from compound 11 to compound 12). Benzoate activation consumes two ATP equivalents in the hydrolysis of one ATP to AMP + 2 P_i. Further energy has to be invested in reversed electron transport steps to allow release of electrons from oxidation reactions of comparably high redox potential [e.g., the pimelyl-CoA dehydrogenase reaction (compound 6 to compound 7) and the glutaryl-CoA dehydrogenase reaction (compound 10 to compound 11)], to be released as molecular hydrogen at hydrogen pressures in the range of 10 Pa, as observed in such cultures (Schöcke and Schink 1997; Schink 1997). In analogy to the butyryl-CoA dehydrogenase reaction in syntrophic butyrate oxidation, these reversed electron transport reactions can be assumed to consume two-thirds of an ATP unit each (Wallrabenstein and Schink 1994). With this, the overall energy balance of benzoate degradation by *S. gentianae* comes to a net energy gain of one-third of an ATP unit per reaction run provided that the dearomatizing benzoyl-CoA reduction does not require any energy input, as hypothesized above. This energy balance agrees with the overall energy balance calculated on the basis of substrate and product concentrations in metabolizing cell suspensions of *S. aciditrophicus* (Warikoo et al. 1996) and *S. gentianae* (Schöcke and Schink 1997), which in both cases came up with a minimum free energy change of –40 kJ per mol benzoate degraded. Thus, these bacteria could synthesize no more than a net amount of one-third to two-thirds of an ATP unit per reaction, and our analysis of reaction steps coupled to ATP expenditure and ATP synthesis balance out to nearly the same overall ATP gain. Future work will have to concentrate on the biochemistry and energetics of the dearomatizing benzoyl-CoA-reducing enzyme of the syntrophically fermenting bacteria.

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