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Anaerobic degradation of *m*-cresol by *Desulfobacterium cetonicum* is initiated by formation of 3-hydroxybenzylsuccinate

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Abstract The anaerobic bacterium *Desulfobacterium cetonicum* oxidized *m*-cresol completely with sulfate as electron acceptor. During growth, 3-hydroxybenzylsuccinate (identified by gas chromatography/mass spectroscopy and by comparison of high-performance liquid chromatography retention time and UV spectrum with a chemically synthesized reference compound) accumulated in the medium. This finding indicates that the methyl group of *m*-cresol is activated by addition to fumarate as in the case of anaerobic toluene metabolism. In cell-free extracts of *D. cetonicum*, the formation of 3-hydroxybenzylsuccinate from *m*-cresol and fumarate was detected at an activity of 0.5 nmol min^{-1} (mg protein)$^{-1}$. This reaction depended strictly on anoxic assay conditions. Treatment with air resulted in a complete loss of activity; however, some activity could be recovered after restoring anoxic conditions. The activity was slightly membrane-associated. 3-Hydroxybenzylsuccinate was degraded via CoA thioesterification and further oxidation to 3-hydroxybenzoyl-CoA as subsequent steps in the degradation pathway.

Key words Anaerobic degradation · Aromatic compounds · *m*-Cresol · Toluene · 3-Hydroxybenzylsuccinate · Sulfate-reducing bacteria · *Desulfobacterium cetonicum*

Introduction

Cresols (*ortho*-, *meta*-, and *para*-methylphenol) are substituted phenols that originate mainly from coal gasification plants, fractionation of coal tar, and petroleum residues, or derive from a variety of synthetic processes. Applications range from use as wood preservatives, disinfectants, and antioxidants to the manufacture of pesticides and cresol-based resins and the production of synthetic vitamin E (Agency for Toxic Substances and Disease Registry 1990). Cresols are considered to be groundwater pollutants and occur in the environment through misuse, accidental spillage, and improper disposal.

Biodegradation under both oxic and anoxic conditions has been shown for all cresol isomers. The aerobic metabolism of cresols has been studied in some detail (Bayly and Barbour 1984; Hopper 1987). The anaerobic metabolism of cresols has been elucidated to a minor extent and in some cases is based only on the detection of potential intermediates in enrichment cultures. *p*-Cresol degradation is initiated by methyl-group oxidation with water as oxygen source (Hopper et al. 1991). *o*-Cresol can be carboxylated to 4-hydroxy-3-methylbenzoate (Bissaillon et al. 1991; Rudolphi et al. 1991) and then be further degraded (Rudolphi et al. 1991). It has also been suggested that *o*-cresol is metabolized by hydroxylation of the methyl group and by further oxidation via 2-hydroxybenzoate, which would be similar to *p*-cresol degradation (Suflita et al. 1989; Schink et al. 1992), but experimental evidence for such a pathway is lacking. Little is known about the pathway of anaerobic *m*-cresol metabolism. There are indications of initiation of degradation via carboxylation in the *para* position to the hydroxy group in methanogenic and sulfidogenic consortia (Roberts et al. 1990; Ramanand and Suflita 1991; Londry and Fedorak 1993) as well as methyl-group oxidation in a nitrate-reducing (Bonting et al. 1995) and in a sulfate-reducing bacterium (Londry et al. 1997). In the latter cases, however, the methyl-group-oxidizing activities could not be demonstrated.

In the present study, we report on *m*-cresol degradation by *Desulfobacterium cetonicum*. This bacterium was originally isolated from oil-field water with butyrate as organic carbon source (Galushko and Rozanova 1991); the utilization of *m*-cresol by *D. cetonicum* has not been previously reported. Biochemical and physiological evidence of degradation of *m*-cresol by this bacterium via 3-hydroxybenzylsuccinate is provided.

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Materials and methods

Organism and culture conditions

D. cetonicum strain 480 (Galushko and Rozanova 1991) was from our own culture collection and was cultivated as described previously (Janssen and Schink 1995). It was grown in 580- or 1200-ml infusion bottles (Müller-Krempel, Bülach, Switzerland) containing 500 or 1000 ml mineral medium under an N_2/CO_2 (80:20, v/v) atmosphere and sealed with butyl-rubber stoppers. The optical density was monitored in 1-cm light path cuvettes at a wavelength of 578 nm. The amount of cell matter formed in growth tests was calculated via a gravimetrically determined conversion factor (OD 0.1 at 578 nm = 18 mg/l). Substrate assimilated into cell material was calculated according to the equation: $C_7H_8O + CO_2 + 3H_2O \rightarrow 2 <C_4H_7O_3>$. Tests for substrate utilization were done in 20-ml Hungate tubes. All tests were run at least in duplicate. Cultures were incubated in the dark at 28 °C.

Preparation of cell suspensions and extracts

Cell suspensions and extracts were obtained under strictly anoxic conditions essentially as described previously (Janssen and Schink 1995) using an anoxic chamber (Coy Laboratory Products, Ann Arbor, Mich., USA). Cells were harvested in the late exponential growth phase, washed, and resuspended in degassed 50 mM potassium phosphate buffer (pH 7.2) amended with 10.0 g NaCl and 1.75 g $MgCl_2 \times 6H_2O$ per liter plus 2.5 mM dithioerythritol (buffer A). For measurements of 3-hydroxybenzylsuccinate synthase activity, the buffer was incubated overnight in the anoxic chamber and supplemented with 0.5 mM titanium(III)NTA (forming buffer B) as a further reducing agent. Cell-free extracts were prepared by French press treatment (138 MPa; one to three passages) followed by two centrifugation steps (15 min; 8000 × *g*; 4 °C); membrane and soluble fractions were separated by ultracentrifugation (1 h at 120,000 × *g*; 4 °C).

Enzyme assays

3-Hydroxybenzylsuccinate synthase activity was measured in buffer B under an N_2 atmosphere in 2-ml Hungate vials at 30 °C. Vials were sealed with butyl-rubber septa. The protein content varied between 1 and 3 mg/ml. Samples were taken with gas-tight syringes (Macherey-Nagel, Düren, Germany) and diluted in ice-cold 100 mM phosphoric acid to stop the reaction. Special care was taken to maintain strictly anoxic conditions throughout the test; all substrate stock solutions were prereduced with 2.5 mM dithioerythritol.

The oxygen sensitivity of 3-hydroxybenzylsuccinate synthase was checked by following activity after a period of O_2 exposure. A gentle air stream was applied over the cell-free extract for 10 min. The test was started by addition of *m*-cresol to the vials. After 30 min, the gas phase was flushed for 5 min with N_2. After an additional 30 min, the assay mix was reduced by addition of Ti(III)NTA (final concentration, 5 mM). Samples were taken throughout the experiment. In a control run, the cell-free extract was flushed with N_2 instead of with air.

m-Cresol methyl-group oxidation was checked for in cell-free extracts in buffer A without a reducing agent. The test was carried out under an N_2 atmosphere in 2-ml Hungate vials, and samples were taken as described for 3-hydroxybenzylsuccinate synthase with NAD(P), FAD, phenazine methosulfate, 2,6-dichloro-phenolindophenol, or methylene blue as potential electron acceptors.

Oxidation of 3-hydroxybenzylsuccinate was measured in buffer B. The assay for 3-hydroxybenzylsuccinate formation was amended with a CoA source and potential electron acceptors at various times during the assay. Succinyl-CoA, acetyl-CoA, or free CoASH plus ATP were tested as CoA sources, and a mixture of NAD, NADP, and FAD was added to the test mixes as electron acceptors (each at a final concentration of 1 mM). Concentrations of substrates and products were monitored discontinuously as described for the assay for 3-hydroxybenzylsuccinate formation. Aliquots of the assays were also analyzed after an alkaline treatment [KOH (pH 12), 80 °C, 20 min]. Dilution of substrate and product concentrations in the assays by addition of a CoA source and electron acceptor was taken into consideration.

Malate dehydrogenase (EC 1.1.1.37) was assayed according to Stams et al. (1984), and fumarate reductase (EC 1.3.1.6) was assayed as described in Beh et al. (1993).

All enzyme activities are expressed as umol substrate transformed per milligram of cell-free extract protein and are the means of at least three determinations.

Analytical procedures

3-Hydroxybenzylsuccinate was identified in cultures grown in 150-ml infusion bottles filled with 60 ml medium. Cells were removed by centrifugation (8000 × *g*, 30 min, 4 °C) after depletion of *m*-cresol in the medium; the supernatant was acidified to pH < 2 by addition of HCl (35%) and was extracted three times with diethyl ether. The extract was concentrated by partial evaporation of the ether at room temperature and was derivatized with ethereal diazomethane (Schwetlick 1970). Nonreacted diazomethane was removed by addition of ethereal acetic acid; the solution was dried at room temperature, and the obtained residue was dissolved in heptane and analyzed by GC-MS. GC-MS analysis was performed with a model HP 6890 GC (Hewlett-Packard, Waldbronn, Germany) equipped with a DB-5 MS fused silica column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm; J&W Scientific, Cologne, Germany) coupled to a HP 5973 series mass selective detector; data were analyzed using Hewlett-Packard G1034C software. The GC oven was programmed from 50 °C (held for 1 min) to 280 °C at 10 °C/min (held for 2 min); the injection port temperature was 250 °C. The gas flow was constant at 3 ml/min. Injections were splitless, with the split turned on after 0.5 min. Spectra were recorded from 50 to 350 mass units at a rate of two scans per second.

Aromatic compounds were quantified by HPLC as described previously (Brune and Schink 1990). Aliphatic acids were measured by HPLC as described by Galushko et al. (1999). Sulfide was determined according to Cline (1969), and protein was determined by the Bradford (1976) method with BSA as the standard.

Synthesis of 3-hydroxybenzylsuccinate

3-Hydroxybenzylsuccinic acid was synthesized according to Stobbe (1911) by condensation of 3-benzyloxybenzaldehyde with diethyl succinate, yielding 3-benzyloxybenzylidene succinic acid, and by subsequent catalytic reduction with H_2. For synthesis of 3-benzyloxybenzylidene succinic acid, 3-benzyloxybenzaldehyde (15 g, 70 mmol) was dissolved in a refluxing solution of sodium methoxide in methanol (10 g sodium in 80 ml MeOH). Diethyl succinate (13.5 g, 80 mmol) was added dropwise within 5 min. The reaction mixture was refluxed on a water bath for 2 h and was then added to 100 ml water. The methanol was removed by evaporation, and the aqueous phase was extracted with diethyl ether to remove the unreacted aldehyde. The aqueous phase was acidified by addition of concentrated HCl (35%). A yellowish product that was recrystallized from acetic acid (yield, 9.1 g; 41%; melting point, 169 °C) precipitated. The product was identified as 3-benzyloxybenzylidene succinic acid by ^{1}H-NMR in CD_3SOCD_3 with spectra collected on a Bruker AC250 instrument (Bruker Analytik, Rheinstetten, Germany). Elemental analysis gave C = 69.4% and H = 5.1%; according to the formula $C_{18}H_{16}O_5$, C = 69.2% and H = 5.1% would have been expected. For synthesis of 3-hydroxybenzylsuccinic acid, the hydrogenating catalyst was prepared by adding solid NaOH to a suspension of 8 g Raney nickel in 50 ml water until no further exothermic reaction could be observed. For hydrogenation and removal of the protecting benzyl residue, 3.5 g

3-benzyloxybenzylidene succinic acid (11 mmol) was dissolved in 40 ml 0.625 M aqueous NaOH. The catalyst was added, and the flask was gassed continuously with hydrogen until no more educt could be detected by thin-layer chromatography (30 h). The catalyst was filtered off, and the filtrate was acidified with 2 M HCl. The reaction mixture was saturated with NaCl and thoroughly extracted with diethylether. Evaporation of the solvent yielded a colorless solid that was recrystallized from water (1.5 g; 68%; melting point, 140 °C). The product was further purified by semipreparative HPLC and was identified by ^{1}H-NMR in CD_3SOCD_3. Elemental analysis gave C = 59.2% and H = 5.0%; according to the formula $C_{11}H_{12}O_5$, C = 58.9% and H = 5.4% would have been expected.

Further chemicals

3-Hydroxybenzoyl-CoA was synthesized according to Wieland [see Decker (1959)] and Merkel et al. (1989) without the final alkali treatment step in 20 mM bicarbonate buffer (pH 7.2) under an N_2/CO_2 atmosphere (80 : 20, v/v). Acetyl-CoA and succinyl-CoA were synthesized from CoASH and the corresponding anhydrides (Simon and Shemin 1953). All CoA esters were purified by HPLC. The CoA esters containing fractions were lyophilized to dryness and dissolved in 10 mM potassium phosphate buffer (pH 6.0). Titanium(III)NTA stock solutions were prepared in an anoxic chamber according to Moench and Zeikus (1983). The solution contained 100 mM Ti^{3+} chelated by 150 mM nitriloacetate. Benzylsuccinate was a gift from F. Widdel (MPI für Marine Mikrobiologie, Bremen, Germany). All other chemicals and gases were of the highest purity available and were obtained from standard commercial sources.

Results

Growth with *m*-cresol

D. cetonicum oxidized *m*-cresol completely to CO_2 with sulfate as electron acceptor; cells grew with a doubling time of 2.8 days. Sulfide was produced concomitantly with substrate utilization and an increase in optical density; sulfide recovery was 98%, expressed as a percentage of theoretical production. After transfer into fresh medium, a long lag phase (up to 7 days) was observed. *D. cetonicum* could tolerate up to 2 mM *m*-cresol in the medium. From the specific growth rate and yield, the in vivo substrate turnover rate was calculated at 8 nmol min^{-1} (mg protein)$^{-1}$. In addition to *m*-cresol, *D. cetonicum* could also use 3-hydroxybenzaldehyde and 3-hydroxybenzoate as electron donor, but not 3-hydroxybenzylalcohol. Oxidation of toluene by *D. cetonicum* had been reported previously (Harms et al. 1999) and was confirmed in this study.

Identification of 3-hydroxybenzylsuccinate in growing cultures of *D. cetonicum*

During growth of *D. cetonicum* with *m*-cresol, a compound that was not observed during growth with other substrates (e.g., 3-hydroxybenzoate) accumulated in the medium was determined using reversed-phase HPLC analysis. This compound was apparently more polar than *m*-cresol as judged from HPLC retention times, and the UV absorption spectrum indicated that this compound contained an aromatic residue (Fig. 1A). Comparison with the retention times and UV spectra of several hypothetical intermediates of anaerobic *m*-cresol degradation indicated that this compound was 3-hydroxybenzylsuccinate. After coinjection of this compound into the HPLC with a reference of 3-hydroxybenzylsuccinate, one symmetrical peak was formed. The identity of 3-hydroxybenzylsuccinate was further confirmed by GC-MS (Fig. 1B,C). 3-Hydroxy-

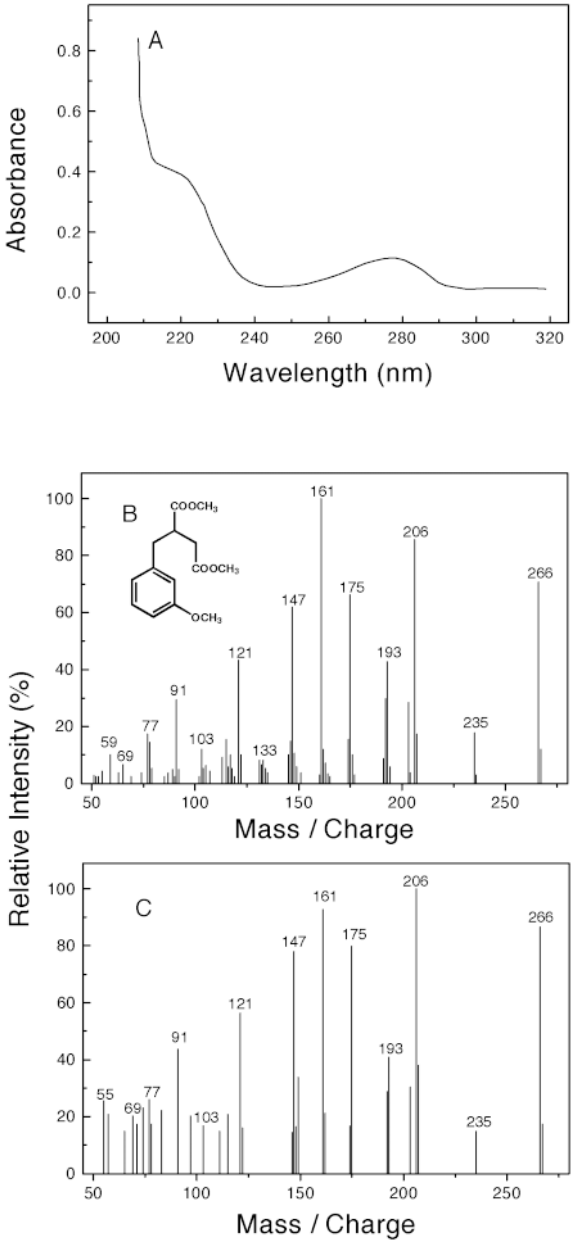

Fig. 1 A–C Identification of 3-hydroxybenzylsuccinate in *m*-cresol-converting cultures of *Desulfobacterium cetonicum*. **A** UV spectrum of 3-hydroxybenzylsuccinate from culture supernatant in acetonitrile (10%) and 10 mM potassium phosphate buffer (pH 2.2). The maximum is at 274 nm. Chemically synthesized 3-hydroxybenzylsuccinate had exactly the same spectrum. Mass analysis by GC-MS of **B** chemically synthesized 3-methoxybenzylsuccinate dimethyl ester and **C** 3-methoxybenzylsuccinate dimethyl ester obtained from culture supernatant. GC retention time was 16 min in both cases; mass units are daltons

benzylsuccinate as a reference substance was chemically synthesized via the Stobbe condensation (see Materials and methods). After the accumulating compound was identified as 3-hydroxybenzylsuccinate, quantitative analysis revealed that it made up 1–3% of the *m*-cresol converted in grown cultures.

We also checked for accumulation of metabolites in the medium during growth on toluene. In cultures pregrown on toluene and fed several times with this substrate, small amounts of benzylsuccinate were identified by coelution with a reference compound in an HPLC run and by comparison of its UV spectrum with that of the reference compound (data not shown).

Formation of 3-hydroxybenzylsuccinate in cell-free extracts

Formation of 3-hydroxybenzylsuccinate during anaerobic *m*-cresol degradation would be analogous to the formation of benzylsuccinate during toluene degradation as reported for denitrifying and sulfate-reducing bacteria (Heider et al. 1999). Hence, we checked cell-free extracts for an activity that added fumarate to the methyl group of *m*-cresol. Formation of 3-hydroxybenzylsuccinate could be measured in crude extract with an activity of 0.5 nmol min^{-1} (mg protein)$^{-1}$ (Fig. 2, Table 1). The activity depended strictly on the addition of *m*-cresol, fumarate, and cell-free extract. No 3-hydroxybenzylsuccinate could be detected in assays free of either *m*-cresol or fumarate, or with heat-denatured extract (90 °C, 10 min). Fumarate could not be substituted by citrate, succinate, or crotonate. The activity was stable for at least several hours at 30 °C. After ultracentrifugation, most of the activity was recovered in the soluble fraction; however, up to 20% of the activity was found in the membrane fraction. After washing of the membrane fraction with 250 mM NaCl and 0.5 mM EDTA in the buffer, the total activity was recovered in the supernatant (Table 1). 3-Hydroxybenzylsuccinate formation depended strictly on anoxic conditions; no activity could be measured in assays under air. However, the 3-hydroxybenzylsuccinate-forming activity was not irreversibly destroyed by molecular oxygen. After removal of O_2 by flushing with N_2 for 5 min and addition of titanium(III)NTA (final concentration, 5 mM), up to 10% of the initial activity could be recovered.

We also checked for possible oxidizing activities acting on *m*-cresol. With NAD(P), FAD, phenazine methosulfate, 2,6-dichloro-phenolindophenol, or methylene blue, no decrease in *m*-cresol concentration was observed in the presence of cell-free extract.

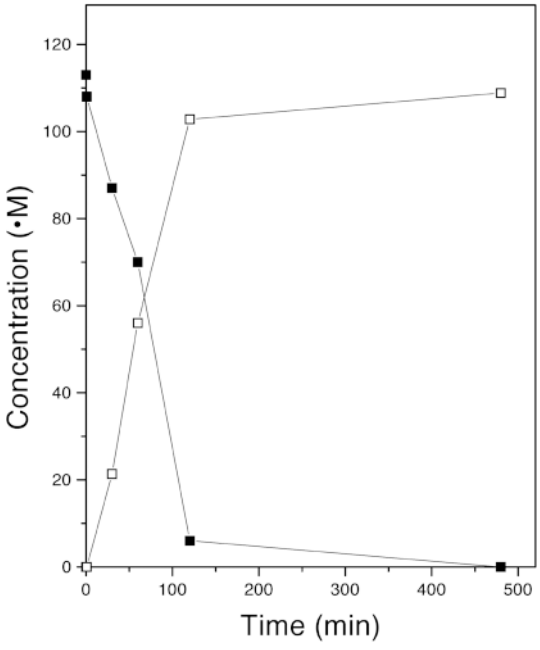

Fig. 2 Conversion of *m*-cresol (■) to 3-hydroxybenzylsuccinate (□) by cell-free extracts of *Desulfobacterium cetonicum*

Catabolism of 3-hydroxybenzylsuccinate

The fate of 3-hydroxybenzylsuccinate was investigated in cell-free extracts. Assays for 3-hydroxybenzylsuccinate formation were amended with a CoA source (succinyl-CoA, acetyl-CoA, or free CoASH and ATP) and/or a mixture of electron acceptors (NAD, NADP, FAD) at various times during the assay. *m*-Cresol concentration and product formation were monitored by HPLC analysis. Without the addition of a CoA source and electron acceptors, the 3-hydroxybenzylsuccinate detected made up more than 96% of the *m*-cresol converted. After addition of succinyl-CoA and electron acceptors to the reaction mix, significantly lower amounts of 3-hydroxybenzylsuccinate

Table 1 Specific activities of 3-hydroxybenzylsuccinate formation from *m*-cresol and fumarate after fractionation of the cell-free extract by ultracentrifugation (1 h at 120,000 × *g*, 4 °C). Malate-dehydrogenase was used as a marker enzyme. After ultracentrifugation, 97% of the total activity was recovered in the supernatant, and < 2% was recovered in the pellet

	Specific activity	Total activity	
	[nmol min^{-1} (mg $protein^{-1}$)]	In fraction (nmol min^{-1})	Relative (%)
Extract	0.5	0.63	100
Supernatant	0.45	0.43	69
Pellet	0.31–0.45	0.09–0.13	14–20
Pellet washed with 250 mM NaCl and 0.5 mM EDTA in the buffer	0	0	0
Washing buffer	1.04	0.06	10

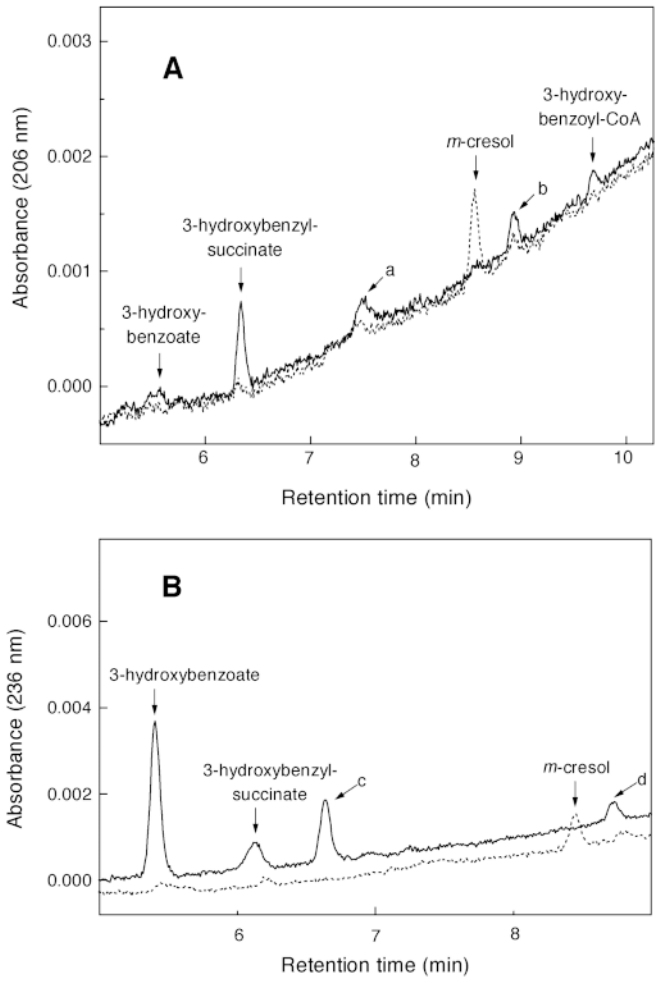

Fig. 3 A–B Products of 3-hydroxybenzylsuccinate oxidation by cell-free extracts of *Desulfobacterium cetonicum*. Sections of HPLC chromatograms of samples after 1 min (*dashed line*) and 120 min (*solid line*) of incubation are shown. Compounds were identified by coelution with standards. **A** Chromatogram before alkaline hydrolysis of the sample. **B** Chromatogram after alkaline treatment of the sample. Peaks marked *a*, *b*, *c*, and *d* were formed independently of the addition of *m*-cresol

were detected (76–82%). Substituting succinyl-CoA with acetyl-CoA or CoASH and ATP in the assays led to higher final concentrations of 3-hydroxybenzylsuccinate (88–91% of the initial *m*-cresol concentration). Similarly, omitting either the CoA source or the electron acceptors resulted in higher concentrations of 3-hydroxybenzylsuccinate as compared to those of assays with both additions (88–95%). After addition of the CoA source and electron acceptors, we detected 3-hydroxybenzoyl-CoA in the reaction mixture (Fig. 3A). The identity of 3-hydroxybenzoyl-CoA was confirmed by detection of 3-hydroxybenzoate after alkaline treatment of the samples (Fig. 3B). The amount of 3-hydroxybenzoyl-CoA (final concentration of up to 20 μM) accounted for most of the difference in concentrations between *m*-cresol converted and 3-hydroxybenzylsuccinate detected. Succinyl-CoA was added to the assays after some conversion of *m*-cresol to 3-hydroxybenzylsuccinate had occurred. This resulted in much higher concentrations of 3-hydroxybenzoyl-CoA detected than in assays in which succinyl-CoA was present in the assay mixture from the beginning. This might be due to rapid hydrolysis of succinyl-CoA by the cell-free extract [22 nmol min^{-1} (mg protein)$^{-1}$]. In addition to 3-hydroxybenzoyl-CoA, small amounts of 3-hydroxybenzoate were detected, probably due to hydrolysis of 3-hydroxybenzoyl-CoA by the cell-free extract. In some assays amended with succinyl-CoA and electron acceptors, at least one additional product accumulated transiently; it had an HPLC retention time (2.9 min) shorter than that of 3-hydroxybenzylsuccinate (6.2 min). This compound was, however, not characterized further because of the lack of standards of the potential products of 3-hydroxybenzylsuccinate oxidation leading to 3-hydroxybenzoyl-CoA.

Influence of added fumarate and other carboxylic acids on *m*-cresol-converting cultures

The influence of fumarate on the degradation of *m*-cresol by growing cultures of *D. cetonicum* was studied. In the presence of fumarate, growth with *m*-cresol was biphasic (Fig. 4). In the first phase, *D. cetonicum* metabolized *m*-cresol at a rate of 9 nmol min^{-1} (mg protein)$^{-1}$. Per mol *m*-cresol, approximately 1 mol fumarate was consumed [8 nmol min^{-1} (mg protein)$^{-1}$] and 0.5 mol succinate accumulated in the medium [5 nmol min^{-1} (mg protein)$^{-1}$]. After *m*-cresol depletion, *D. cetonicum* grew at the expense of fumarate [12 nmol min^{-1} (mg protein)$^{-1}$]. Succinate was formed at a lower rate [0.04 nmol min^{-1} (mg protein)$^{-1}$] as compared to that of the first growth phase. Malate accumulated transiently after depletion of *m*-cresol in the medium (not shown). In growth experiments with succinate or acetate in the presence of *m*-cresol,

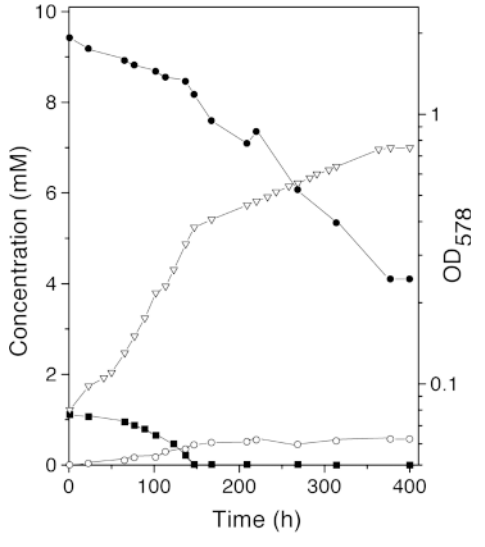

Fig. 4 Degradation of *m*-cresol (■) in the presence of fumarate (●). Formation of succinate (○) and increase in OD (▽) are shown

Table 2 Growth rates and yields of *Desulfobacterium cetonicum* grown on *m*-cresol in the presence of aliphatic acids. Yields were calculated from OD_{578} values directly after *m*-cresol depletion in the medium via an experimentally determined conversion factor

Growth substrate	Growth rate (day^{-1})	Growth yield [g (dry wt.) × (mol *m*-cresol)$^{-1}$]
m-Cresol alone	0.24	34.3
m-Cresol plus fumarate	0.31	38
m-Cresol plus succinate	0.26	36.2
m-cresol plus acetate	0.27	35.9

growth rates and yields were significantly lower than those of cultures amended with fumarate, albeit higher than those without addition of an aliphatic acid (Table 2). During growth on the putative intermediate 3-hydroxybenzoate, there was no growth stimulation by fumarate as compared to controls with succinate or acetate. *D. cetonicum* did not grow with *m*-cresol or 3-hydroxybenzoate as electron donor and fumarate as sole potential electron acceptor, and fumarate reductase was not detected in *D. cetonicum*.

Discussion

D. cetonicum could grow by complete oxidation of *m*-cresol with sulfate as electron acceptor. The substrate was converted according to the following equation [$\Delta G^{\circ\prime}$ value calculated according to Thauer et al. (1977)]:

$$4C_7H_8O + 17SO_4^{2-} + 17H^+ \rightarrow 28CO_2 + 17HS^- + 16H_2O$$

$\Delta G^{\circ\prime}$ = –225 kJ/mol *m*-cresol.

During growth, small amounts of 3-hydroxybenzylsuccinate were excreted into the medium, corresponding to 1–3% of the *m*-cresol converted. Identification of 3-hydroxybenzylsuccinate as a potential degradation intermediate led to the hypothesis that *m*-cresol was degraded through a pathway similar to that described for anaerobic degradation of toluene. In this pathway, the first step is the formation of benzylsuccinate by addition of fumarate to the methyl group of toluene [reviewed in Heider et al. (1999)]. A possible similarity between initial steps of anaerobic *m*-cresol degradation and toluene degradation has also been recently suggested by Heider and Fuchs (1997) and Harwood et al. (1999).

Experiments were carried out to check for formation of 3-hydroxybenzylsuccinate from *m*-cresol and fumarate in cell-free extracts of *D. cetonicum*. We found such activities at 0.5 nmol min^{-1} (mg protein)$^{-1}$, which is approximately 7% of the in vivo transformation activity. 3-Hydroxybenzylsuccinate synthase was the only enzyme detected that acted on the *m*-cresol methyl group; no hydroxylating activity was observed. At the present time, nothing is known about the reaction mechanism of 3-hydroxybenzylsuccinate formation. One can envision that it proceeds in a manner similar to the formation of benzylsuccinate from toluene and fumarate because in both cases a nonactivated hydrogen atom has to be abstracted from a methyl group to form a new C–C bond. There is strong evidence that benzylsuccinate formation from fumarate and toluene is accomplished in nitrate-reducing bacteria via formation of an enzyme-bound radical (Beller and Spormann 1997, 1998; Coschigano et al. 1998; Leuthner et al. 1998). Benzylsuccinate synthase is extremely oxygen-sensitive and is irreversibly destroyed by oxygen due to cleavage of the radical-harboring peptide chain (Leuthner et al. 1998). Considering a mechanism of 3-hydroxybenzylsuccinate formation in *D. cetonicum* similar to that of benzylsuccinate generation, it is not surprising that this reaction also required strictly anoxic conditions; no activity was measured in assays under air. However, after removal of O_2, some activity was recovered. Perhaps 3-hydroxybenzylsuccinate synthase exists in two forms: an active one that is oxygen-sensitive, and an inactive one that is oxygen-insensitive. During preparation of cell-free extract and in assays under air, part of the inactive form could persist and be converted to the active form after restoring reducing conditions. This would resemble the inactivation/activation transition of pyruvate formate lyase or of anaerobic ribonucleotide reductase in *Escherichia coli* (Knappe and Sawers 1990; Sun et al. 1995).

D. cetonicum can also grow with toluene (Harms et al. 1999). After several additions of toluene to growing cultures of *D. cetonicum*, we found that benzylsuccinate accumulated in the growth medium, indicating that in this strain toluene is degraded as has been described for other anaerobic bacteria. It remains to be elucidated whether formation of 3-hydroxybenzylsuccinate and benzylsuccinate is catalyzed by the same or by two different enzymes.

A hypothetical pathway for the further conversion of 3-hydroxybenzylsuccinate is depicted in Fig. 5. It is analogous to the proposed conversion of benzylsuccinate to benzoyl-CoA in denitrifying bacteria of the genus *Thauera* (Heider et al. 1999). Evidence for this pathway of 3-hydroxybenzylsuccinate conversion comes from two different experiments:

1. 3-Hydroxybenzylsuccinate is converted to 3-hydroxybenzoyl-CoA in cell-free extracts in the presence of the electron acceptors NAD(P) and FAD, and a CoA source. Besides 3-hydroxybenzoyl-CoA, an additional product whose nature remains to be elucidated accumulated transiently. The highest concentrations of 3-hydroxybenzoyl-CoA were found with succinyl-CoA, indicating that this is the physiological CoA donor. For benzylsuccinate oxidation to benzoyl-CoA in *Thauera aromatica*, involvement of a benzylsuccinate:succinyl-CoA CoA transferase has already been reported (Heider et al. 1999).

2. Further evidence for conversion of 3-hydroxybenzylsuccinate to 3-hydroxybenzoyl-CoA as outlined in Fig. 5 comes from experiments with supplementation of fumarate to *m*-cresol converting cultures. Per mol of *m*-cresol degraded, approximately 1 mol of fumarate was consumed, and close to 1 mol of succinate was excreted

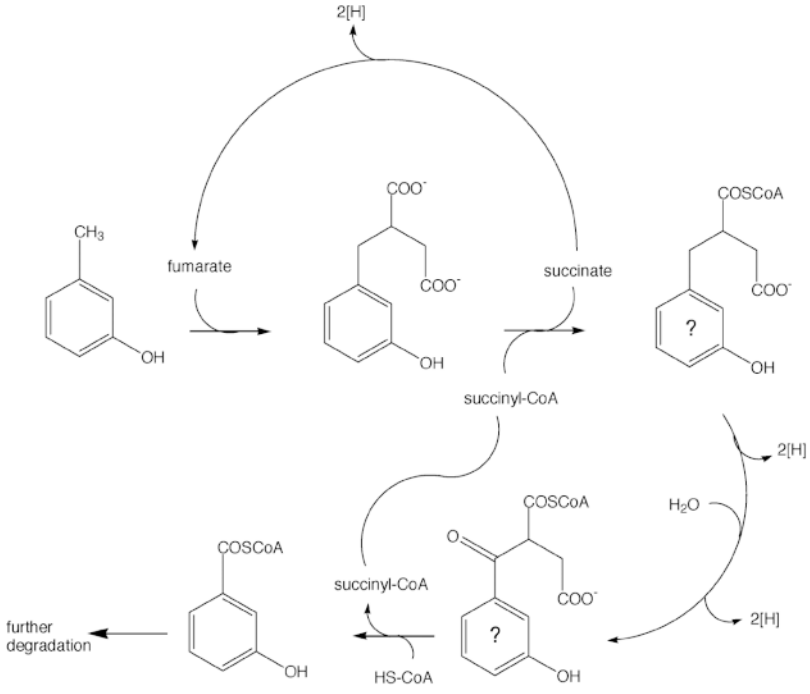

Fig. 5. Proposed pathway of *m*-cresol degradation by *Desulfobacterium cetonicum*. Compounds denoted with a *question mark* (3-hydroxybenzylsuccinate-CoA and 3-hydroxybenzoylsuccinyl-CoA) were not identified

into the medium (Fig. 4). This conversion of fumarate to succinate was observed only during growth with *m*-cresol; with the presumed intermediate 3-hydroxybenzoate, no fumarate consumption was detected. Assuming a pathway of *m*-cresol degradation as outlined in Fig. 5, succinate has to be reoxidized to fumarate for 3-hydroxybenzylsuccinate formation. This oxidation is thermodynamically difficult for sulfate-reducing bacteria. One way to accomplish this oxidation could be to involve an energy-driven reversed electron-transport (Schirawski and Unden 1998). Such energy investment is not necessary if externally provided fumarate is used for the 3-hydroxybenzylsuccinate synthase reaction. The increase of both growth rate and yield (Table 2) upon addition of fumarate strongly supports the assumption of an energy-dependent oxidation of succinate to fumarate during growth on *m*-cresol. It is important to note that fumarate could not be used as terminal electron acceptor by *D. cetonicum*, and no fumarate reductase activity could be measured.

The further fate of 3-hydroxybenzoyl-CoA in *D. cetonicum* is not yet known. Reductive dehydroxylation to benzoyl-CoA might occur as exemplified in *Sporotomaculum hydroxybenzoicum* (J.A. Müller and B. Schink, unpublished work), and benzoyl-CoA could be further converted to aliphatic products (Harwood et al. 1999). Use of benzoate as growth substrate by *D. cetonicum* has been reported previously (Galushko and Rozanova 1991).

Two alternative ways of initiating anaerobic *m*-cresol degradation have been discussed in the literature: (1) methyl group hydroxylation forming 3-hydroxybenzylalcohol and further oxidation to 3-hydroxybenzoate, and (2) *para*-carboxylation yielding 4-hydroxy-2-methylbenzoate. The first hypothesis is based mainly on the identification of 3-hydroxybenzoate as an intermediate of *m*-cresol degradation by denitrifying and sulfate-reducing bacteria (Bonting et al. 1995; Londry et al. 1997). The respective *m*-cresol hydroxylating activities, however, could not be shown. The finding of 3-hydroxybenzoate as an intermediate can also be explained by our pathway and, therefore, cannot be regarded as evidence for methyl group hydroxylation. In methanogenic and sulfidogenic consortia, *m*-cresol appears to be converted via a carboxylation reaction (Roberts et al. 1990; Ramanand and Suflita 1991). In both cases, 4-hydroxy-2-methylbenzoate is formed during *m*-cresol degradation. It can be hypothesized that fermenting bacteria degrade *m*-cresol via a pathway not involving 3-hydroxybenzylsuccinate formation in order to encompass the energetically unfavorable oxidation of succinate to fumarate. A similar phenomenon of diverse degradation routes of aromatic compounds depending on the terminal electron-accepting system has also been shown for anaerobic resorcinol degradation (Kluge et al. 1990; Philipp and Schink 1998).

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