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Ralf Rabus · Johann Heider

Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria

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Abstract The initial activation reactions of anaerobic oxidation of the aromatic hydrocarbons toluene and ethylbenzene were investigated in cell extracts of a toluene-degrading, sulfate-reducing bacterium, *Desulfobacula toluolica*, and in cell extracts of strain EbN1, a denitrifying bacterium capable of degrading toluene and ethylbenzene. Extracts of toluene-grown cells of both species catalysed the addition of fumarate to the methyl group of [*phenyl*-^{14}C]-toluene and formed [^{14}C]-labeled benzylsuccinate. Extracts of ethylbenzene-grown cells of strain EbN1 did not catalyse this reaction, but catalysed the formation of 1-phenylethanol and acetophenone from [*methylene*-^{14}C]-ethylbenzene. Toluene-grown cells of *D. toluolica* and strain EbN1 synthesised highly induced polypeptides corresponding to the large subunits of benzylsuccinate synthase from *Thauera aromatica*. These polypeptides were absent in strain EbN1 after growth on ethylbenzene, although a number of different polypeptides were highly induced. Thus, formation of benzylsuccinate from toluene and fumarate appears to be the general initiating step in anaerobic toluene degradation by bacteria affiliated with the phylogenetically distinct β-subclass (strain EbN1 and *T. aromatica*) and δ-subclass (*D. toluolica*) of the Proteobacteria. Anaerobic ethylbenzene oxidation proceeds via a different pathway involving a two-step oxidation of the methylene group to an alcohol and an oxo group; these steps are most probably followed by a biotin-independent carboxylation reaction and thiolytic cleavage.

R. Rabus[1]
Max-Planck-Institut für marine Mikrobiologie, Celsiusstrasse 1, D-28359 Bremen, Germany

J. Heider (✉)
Universität Freiburg, Institut für Biologie II, Mikrobiologie, Schänzlestrasse 1, D-79104 Freiburg, Germany
e-mail: heiderj@ruf.uni-freiburg.de
Tel. +49-761-203-2774; Fax +49-761-203-2626

Present address:
[1] University of California San Diego, Department of Biology, La Jolla, CA 92093–0116, USA

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Introduction

Bacterial metabolism of aromatic hydrocarbons has long been considered to be an absolutely oxygen-dependent process. Only the last decade of microbiological research has proven that these compounds are also utilised as carbon and energy sources by anaerobic bacteria. Readily degradable substrates under anoxic conditions include toluene, ethylbenzene, xylenes and other alkylbenzenes (Rabus et al. 1996). Bacterial species capable of anaerobic degradation of alkylbenzenes belong to four physiological groups: denitrifying bacteria of the β-subclass of Proteobacteria, sulfate- and ferric-iron-reducing bacteria of the δ-subclass, and fermentative bacteria in syntrophic methanogenic or sulfidogenic associations [reviewed in Heider and Fuchs (1997)]. Well-characterised bacteria capable of anaerobic degradation of toluene include the ferric-iron-reducing *Geobacter metallireducens* (Lovley and Lonergan 1990), several isolates of related denitrifying species represented by *Thauera aromatica* and *Azoarcus tolulyticus* (Dolfing et al. 1990; Altenschmidt and Fuchs 1991; Evans et al. 1991; Schocher et al. 1991; Fries et al. 1994; Rabus and Widdel 1995 a), and the sulfate-reducing species *Desulfobacula toluolica* and strain PRTOL1 (Rabus et al. 1993; Beller et al. 1996). The denitrifying strains EbN1 and EB1 are the first pure cultures shown to be capable of anaerobic degradation of ethylbenzene (Rabus and Widdel 1995 a; Ball et al. 1996). Strain EbN1 is the only pure culture that can utilise either toluene or ethylbenzene as sole substrate.

In all aerobic hydrocarbon-utilizing organisms, catabolic reactions for the initial attack at the hydrocarbons depend on molecular oxygen as cosubstrate. In contrast, under anoxic conditions, the initial attack at the hydrocar-

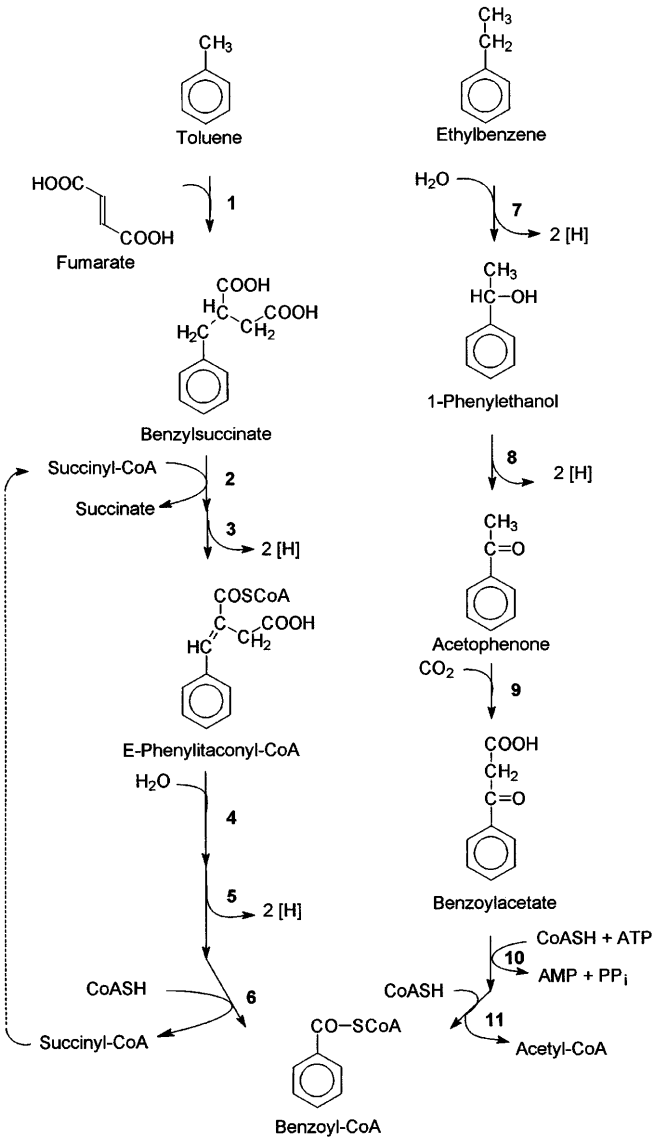

Fig. 1 Proposed metabolic pathways of anaerobic degradation of toluene and ethylbenzene. Enzymes involved in toluene metabolism are represented by *numbers 1–6*, those catalysing the proposed steps in ethylbenzene metabolism by *7–11*. Note that the carboxylation step (*9*) may require energy; the proposed activation of benzoylacetate to the CoA thioester (*10*) may be catalysed either by a CoA ligase or a CoA transferase

bon must proceed via oxygen-independent reactions. The pathways of anaerobic toluene and ethylbenzene degradation in denitrifying bacteria lead to benzoyl-CoA as the last aromatic intermediate (Fig. 1). At this point, the anaerobic metabolic pathways of most aromatic compounds converge. The dearomatisation of the aromatic ring in the absence of oxygen is accomplished by ATP-dependent reduction of benzoyl-CoA to a cyclic diene derivative, as catalysed by benzoyl-CoA reductase (Boll and Fuchs 1995, 1997).

The available data on anaerobic degradation of toluene and ethylbenzene in denitrifying bacteria suggest that different pathways are employed for the two alkylbenzenes. The initiation reaction of anaerobic toluene oxidation is well-documented with whole and permeabilised cells, cell extracts, and the purified enzyme. The first step is the formal addition of the cosubstrate fumarate to the methyl group of toluene, catalysed by the glycyl-radical enzyme benzylsuccinate synthase (Biegert et al. 1996; Beller and Spormann 1997a; Leuthner et al. 1998). The product formed, benzylsuccinate, is then further oxidised to benzoyl-CoA (Fig. 1; Biegert et al. 1996; C. Leutwein, B. Leuthner and J. Heider, unpublished work). Benzylsuccinate synthase from *T. aromatica* has been purified and characterised. The enzyme contains a glycyl radical close to the carboxy terminus of the large subunit. The polypeptide backbone of the glycyl-radical-containing protein is cleaved at the radical site upon contact with oxygen. Because only one of two subunits in the holoenzyme contains a radical, a characteristic double band corresponding to the toluene-induced enzyme is visible in cell extracts separated on SDS-polyacrylamide gels (Leuthner et al. 1998). The fate of ethylbenzene in strains EbN1 and EB1 has been inferred from growth experiments and substrate conversion tests with whole cells (Rabus and Widdel 1995a; Ball et al. 1996). First ethylbenzene appears to be oxidised at the methylene group, yielding 1-phenylethanol and acetophenone (Fig. 1, enzymes 7 and 8). Acetophenone is then thought to be carboxylated at the methyl group (Fig. 1, enzyme 9); the resulting benzoylacetate would be activated to a CoA thioester and cleaved to benzoyl-CoA and acetyl-CoA by a β-keto thiolase (Fig. 1, enzymes 10 and 11).

In the present study, we investigated the initial reactions of anaerobic toluene oxidation in the sulfate-reducing *D. toluolica* and the nitrate-reducing strain EbN1 in vitro. We demonstrate that both bacteria form [^{14}C]-labeled benzylsuccinate from [^{14}C]-labeled toluene and appear to synthesise an inducible benzylsuccinate synthase when grown on toluene. The implementation of this pathway by phylogenetically distant bacteria indicates that addition of fumarate to the methyl group is a generally conserved pathway used to attack toluene under anoxic conditions. We also show that anaerobic degradation of ethylbenzene in extracts of denitrifying bacteria is indeed initiated via direct oxidation of the methylene group, yielding 1-phenylethanol and acetophenone. The presence of two distinct and substrate-inducible sets of enzymes for degradation of ethylbenzene and toluene in strain EbN1 is demonstrated.

Materials and methods

Cell growth and preparation of extracts

Sulfate-reducing *D. toluolica* strain Tol2 (DSM 7467; Rabus et al. 1993) and denitrifying strain EbN1 (Rabus and Widdel 1995b) were isolated from enrichment cultures on toluene and ethylbenzene, respectively, and have been subcultured in the laboratory since then. *D. toluolica* was grown anaerobically in defined salt-water medium containing 1 mM Na_2S. Toluene was provided in an inert carrier phase of 40 ml 2,2,4,4,6,8,8-heptamethylnonane/l medium (2% toluene, v/v), as previously described (Rabus et al. 1993; Rabus and Widdel 1995b). Cultures were harvested under

strictly anoxic conditions at an optical density (660 nm) of 0.2, which corresponded to the mid-exponential growth phase. The centrifuged cells were washed once with sulfide-reduced saltwater medium, shock-frozen in liquid N_2, and stored at –80 °C. Three grams (wet mass) of cells were obtained from 22 l of culture. The denitrifying strain EbN1 was grown in 2-l bottles on a defined, ascorbate (4 mM)-reduced mineral medium with either toluene or ethylbenzene as sole carbon and energy source (Rabus and Widdel 1995 a). Conditions for growth and harvesting of cells were as described by Rabus and Widdel (1995 a). Typical cell yields were 0.2 g (wet mass)/l culture harvested at an OD_{660} of 0.24. A 200-l culture of strain EbN1 was grown on ethylbenzene (4% in 3 l of mineral oil) in mineral medium containing 10 mM Na-nitrate as described by Biegert et al. (1996). When nitrate and ethylbenzene were depleted, these compounds were resupplied to the fermenter at the initial concentrations. Exponential growth was obtained up to an OD_{660} of 1.5, and the cell yield after harvesting was 180 g (wet mass).

Crude extracts were prepared under strictly anoxic conditions. Cells were resuspended in 1 vol. anoxic buffer [200 mM Tris-HCl, 2 mM $MgCl_2$, 20% glycerol and 0.5 mM dithionite (pH 8.0)] and were broken by one passage through a French press cell. Cell debris and unbroken cells were removed from the extract by centrifugation (1 h at 20,000 × *g*). The small amount of available toluene-grown cells of strain EbN1 necessitated the use of 3 vol. of buffer for extract preparation. In this case, we added 5 mM Na-fumarate to the buffer since the presence of this cosubstrate was found to stabilise benzylsuccinate synthase from *T. aromatica* against inactivation (H. Schulz and J. Heider, unpublished work).

Enzyme assays

Formation of products from toluene or ethylbenzene was assayed at 30°C in cell extracts by determining the production of [^{14}C]-labeled products from [*phenyl*-^{14}C]-toluene and [*methylene*-^{14}C]-ethylbenzene. Cell extract (300 µl) was transferred into an anoxic glass vial, and cosubstrates were added from anoxic stock solutions, each to a final concentration of 2 mM (fumarate, coenzyme A, and nitrate or phenazine methosulfate/sulfite for toluene conversion reactions; and nitrate, bicarbonate, ATP and coenzyme A for ethylbenzene conversion reactions). The reactions were started by adding 75 kBq of [*phenyl*-^{14}C]-toluene in isopropanol (final concentration, 120 µM) or 8 kBq [*methylene*-^{14}C]-ethylbenzene (final concentration, 1.1 mM). Aliquots of 50 µl were taken after various incubation times. The reaction was stopped by adding 5 µl of a 50% H_2SO_4 solution; the precipitated proteins were removed by centrifugation, and the amount of nonvolatile radioactivity formed was analysed by liquid scintillation counting. The samples and appropriate reference compounds were also applied on TLC plates (silica gel 60, Merck) and developed with benzene/dioxan/acetic acid (80 : 10 : 10, by vol.). Radioactive compounds formed from [^{14}C]-toluene or [^{14}C]-ethylbenzene were detected by autoradiography. Nonlabeled reference compounds were detected on TLC plates by fluorescence-quenching.

Other methods

Proteins were separated by SDS-PAGE on 11% (w/v) polyacrylamide gels as described by Laemmli (1970). Molecular size markers

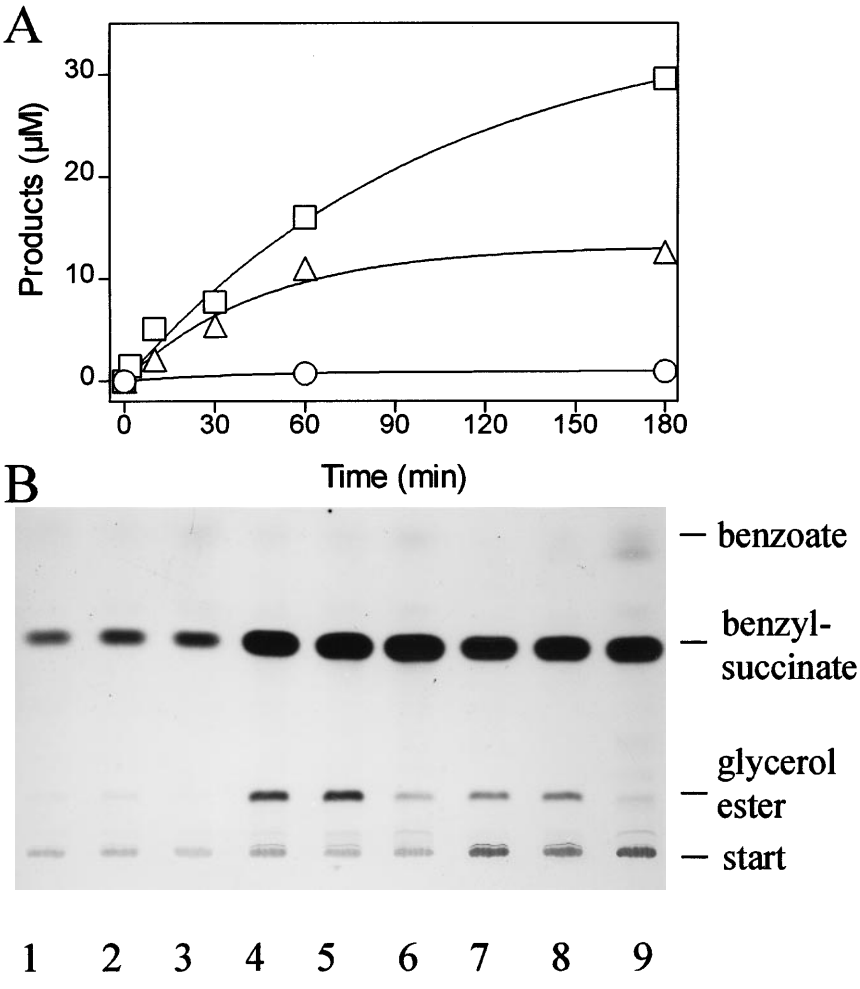

Fig. 2 A, B Anaerobic conversion of [*phenyl*-^{14}C]-toluene to nonvolatile products by cell-free extracts of toluene-grown, sulfate-reducing *Desulfobacula toluolica*. **A** Time course of product formation in assays without supplementation (○), with addition of fumarate (□), and with addition of fumarate, CoASH, and electron acceptors (△). **B** TLC analysis of reaction products formed. The migration positions of benzoate and benzylsuccinate are indicated; a benzylsuccinate derivative generated during sample preparation and tentatively identified as glycerol ester of benzylsuccinate is also indicated. Benzoyl-CoA stays at the application line under the conditions employed. *Lanes 1–3* assay without supplementation after 1, 3 and 12 h; *4–6* assay with added fumarate after 1, 3 and 12 h; and *7–9* assay with added fumarate, CoASH, phenazine methosulfate and sulfite (2 mM each) after 1, 3 and 12 h

used were phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), lactate dehydrogenase (34 kDa) and carboanhydrase (29 kDa). Biotin-containing proteins in cell extracts were detected by separating the proteins by SDS-PAGE and electroblotting them on nitrocellulose sheets (Schleicher & Schüll, Dassel, Germany) following a modification of standard methods (Ausubel et al. 1987). The filters were blocked with 5% (w/v) skim milk powder in basal buffer [50 mM Tris-HCl (pH 7.5) containing 0.9% NaCl], washed, and treated with avidin coupled to horseradish peroxidase (8.5 μg ml^{-1} of basal buffer). After washing, biotin-containing polypeptides were detected with freshly mixed solutions A [10 ml of 3 mg 4-chloronaphtol (ml methanol)$^{-1}$ and B (50 ml of 0.02% H_2O_2 in basal buffer). Protein concentrations were measured according to Bradford (1976) with bovine serum albumin as the standard.

Materials

Chemicals were obtained from Aldrich (Steinheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany), or Roth (Karlsruhe, Germany). Biochemicals were obtained from Boehringer (Mannheim, Germany) or Gerbu (Gaiberg, Germany); gases were from Linde (Höllriegelskreuth, Germany). [*phenyl*-^{14}C]-Toluene (specific radioactivity, 2 GBq/mmol) and [*methylene*-^{14}C]-ethylbenzene (specific radioactivity, 218 MBq/mmol) were from American Radiolabeled Compounds/Biotrend (Köln, Germany).

Results

Toluene degradation by sulfate-reducing *D. toluolica*

Extracts of toluene-grown *D. toluolica* cells catalysed the formation of [*phenyl*-^{14}C]-benzylsuccinate from [*phenyl*-^{14}C]-toluene and fumarate. Benzylsuccinate [and its glycerol-ester; see Fig. 2 and Biegert et al. (1996)] were the only detectable products when no other substrates were present (Fig. 2). Values of 30–50 pmol min^{-1} (mg protein)$^{-1}$ were determined for the initial velocity of benzylsuccinate formation in different extracts. In the absence of added fumarate, benzylsuccinate was formed at a 40-fold lower rate (Table 1; Fig. 2). Fumarate was probably supplied from endogenous sources in these experiments, as previously observed in extracts of toluene-grown *T. aromatica* (Biegert et al. 1996). In order to investigate whether benzylsuccinate was further oxidised to benzoyl-CoA, coenzyme A and sulfite plus phenazine methosulfate (acting as potential electron acceptors) were added to the assays. The initial velocity of toluene conversion to products decreased (Table 1), but benzylsuccinate remained the principal product under these conditions, even after prolonged incubation. However, increased formation of benzoyl-CoA in these assays indicated some degree of further oxidation (Fig. 2). The highest specific activity obtained in cell extracts was only 0.3% of the velocity of toluene degradation by whole cells [18 nmol min^{-1} (mg protein)$^{-1}$; see Rabus et al. (1993)], or 10% of that obtained with permeabilised cells of strain PRTOL1, a different toluene-degrading sulfate reducer (Beller and Spormann 1997b). This is reminiscent of the difference in activity values measured with cell extracts or permeabilised cells of denitrifying toluene-degrading bacteria (Biegert et al. 1996; Beller and Spormann 1997a). It may be attributed either to the extreme oxygen sensitivity of benzylsuccinate synthase (Leuthner et al. 1998) or to the loss of an activating factor or additional cosubstrate for the enzyme upon extract preparation.

Benzyl alcohol (10 mM) and benzaldehyde (10 mM) inhibited synthesis of benzylsuccinate from toluene in extracts of both *D. toluolica* and *T. aromatica* (data not shown). This inhibition was reversible in *T. aromatica* ex-

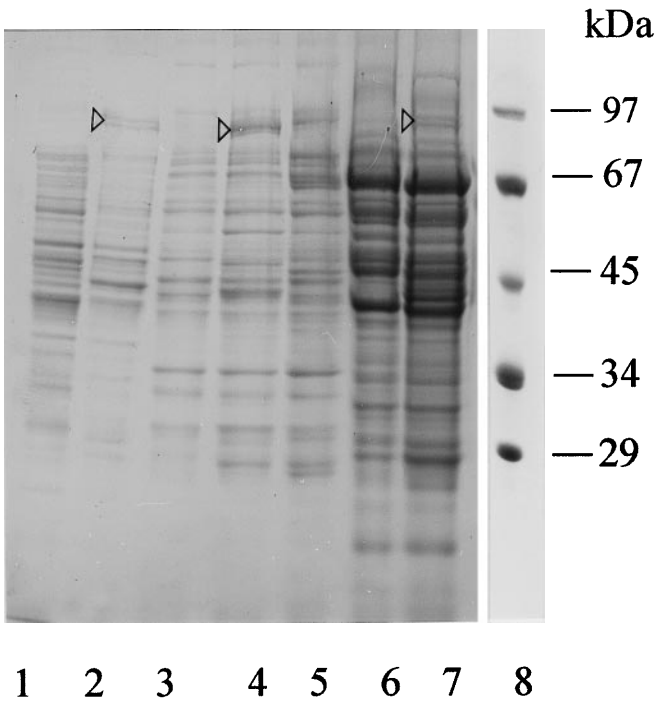

Fig. 3 Polypeptide patterns of cells grown on different substrates. Cell extracts were separated by SDS-PAGE on an 11% (w/v) gel; the resolved proteins were stained with Coomassie blue. Migration positions of the large subunits of benzylsuccinate synthase of *Thauera aromatica* (apparent masses, 94 and 90 kDa) and the comigrating polypeptides in the other species are indicated by *arrows*. Amount of protein applied: 15 μg per lane for *T. aromatica* and strain EbN1, and 50 μg per lane for *Desulfobacula toluolica*, to account for the lower amount of induced proteins in this species. *Lanes*: denitrifying *T. aromatica* grown on benzoate (*1*) and toluene (*2*); denitrifying strain EbN1 grown on benzoate (*3*), toluene (*4*) and ethylbenzene (*5*); sulfate-reducing *D. toluolica* grown on benzoate (*6*) and toluene (*7*); standard proteins (*8*)

Table 1 Initial rates of toluene conversion into products [pmol min^{-1} (mg protein)$^{-1}$] in extracts of toluene-grown cells of sulfate-reducing *Desulfobacula toluolica*, and toluene- and ethylbenzene-grown cells of denitrifying strain EbN1 (*nd* not determined)

Supplements added (2 mM each)	*D. toluolica*	Strain EbN1 (Toluene)	Strain EbN1 (Ethylbenzene)
None	0.9 ± 0.2	nd	nd
Fumarate	39 ± 11	4.0 ± 0.4	< 0.3
Fumarate, CoASH, phenazine methosulfate, sulfite	19 ± 8	nd	nd

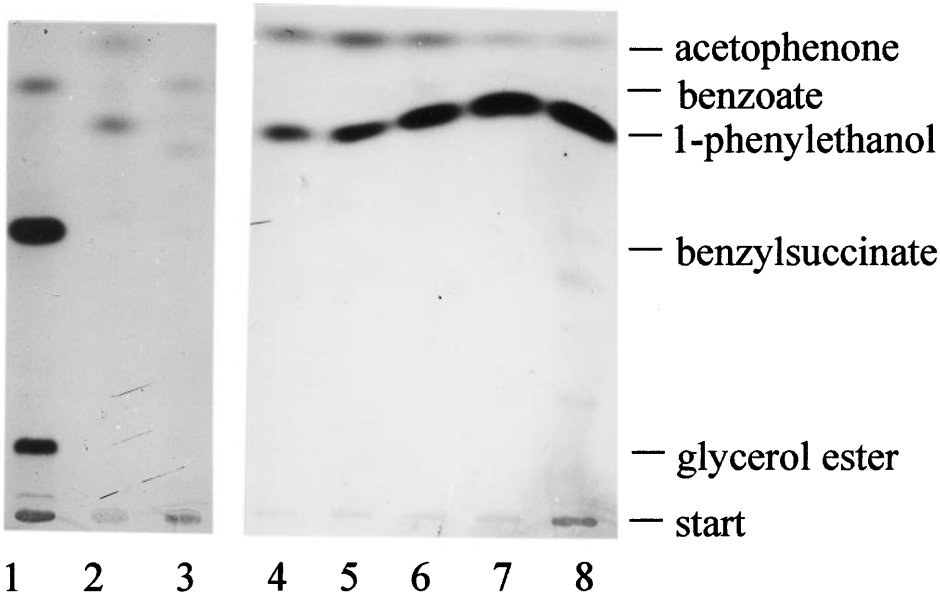

Fig. 4 TLC analysis of transformation products of [*phenyl*-^{14}C]-toluene and [*methylene*-^{14}C]-ethylbenzene by denitrifying strain EbN1. *Lanes 1, 2,* experiments with extracts of toluene-grown cells. Overnight incubation with [^{14}C]-toluene in the presence of fumarate, CoASH and nitrate (2 mM each; *lane 1*) and with [^{14}C]-ethylbenzene in the presence of fumarate, CoASH, bicarbonate, ATP and nitrate (2 mM each; *lane 2*). *Lanes 3–8* experiments with extracts of ethylbenzene-grown cells: 2-h incubation with [^{14}C]-toluene in the presence of fumarate, CoASH and nitrate (2 mM each; *lane 3*) and time course of product formation from [^{14}C]-ethylbenzene in the presence of nitrate, bicarbonate, ATP and CoASH (2 mM each; *lanes 4–8*). Samples were withdrawn after incubation for 5 min (*lane 4*), 15 min (*lane 5*), 30 min (*lane 6*), 60 min (*lane 7*), and overnight (*lane 8*). Migration positions of acetophenone, benzoate, 1-phenylethanol, benzylsuccinate and its glycerol ester are shown; benzoyl-CoA remains at the starting point

tracts, which oxidised benzyl alcohol to benzoate after 2 h via benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase (Biegert et al. 1996), but not in *D. toluolica* extracts. This observation is in accordance with the reported irreversible inhibition of toluene oxidation by benzyl alcohol in cell suspensions of *D. toluolica* (Rabus et al. 1993). Strong similarity of the benzylsuccinate synthases of the two species is also indicated by SDS-PAGE analysis of the protein patterns. Toluene-grown cells of *D. toluolica* contain two polypeptides of approximately the same masses of the α-subunit of benzylsuccinate synthase and its oxygenolytic truncation product (α′); these are lacking in benzoate-grown cells (Fig. 3). In addition to the large subunits of benzylsuccinate synthase, other toluene-induced polypeptides of *D. toluolica* (e.g. at 45 kDa) appear to correspond to analogous proteins of toluene-grown *T. aromatica* (Fig. 3; Heider et al. 1998).

Toluene degradation by denitrifying strain EbN1

The denitrifying strain EbN1 was grown anaerobically on toluene or ethylbenzene as sole carbon and energy source. Both cell batches were analysed for in vitro transformation of [*phenyl*-^{14}C]-toluene plus fumarate to benzylsuccinate. Extracts of toluene-grown cells were found to convert labeled toluene to nonvolatile products at a rate of 3.4–4.3 pmol min^{-1} (mg protein)$^{-1}$. Equally low activities have been measured in some similarly prepared *T. aromatica* extracts, possibly reflecting sensitivity of benzylsuccinate synthase towards dilution (C. Leutwein and J. Heider, unpublished work). The [^{14}C]-labeled products formed in these experiments were identified by TLC and autoradiography. As expected, the products formed from toluene in extracts of toluene-grown cells were benzylsuccinate and its glycerol ester (Fig. 4). No significant further oxidation of benzylsuccinate to benzoyl-CoA was recorded, even when CoASH and nitrate were added to the assay (data not shown). A similar effect was also found in *T. aromatica* extracts, which did not catalyse oxidation of benzylsuccinate to benzoyl-CoA at low protein concentrations (Biegert et al. 1996). Extracts of ethylbenzene-grown cells only exhibited background activities of < 0.3 pmol min^{-1} (mg protein)$^{-1}$ with [*phenyl*-^{14}C]-toluene plus fumarate (Table 1). No [^{14}C]-labeled benzylsuccinate was produced from [*phenyl*-^{14}C]-toluene in these experiments, but a very slow accumulation of [^{14}C]-labeled benzoate was observed (Fig. 4). This may suggest some degree of slow, cometabolic oxidation of toluene via the ethylbenzene degradation pathway.

SDS-PAGE of cell extracts of strain EbN1 grown on benzoate, toluene and ethylbenzene showed different polypeptide patterns after growth on the different substrates (Fig. 3). Bands of highly induced proteins comigrating with the α/α' doublet of benzylsuccinate synthase of *T. aromatica* were visible in toluene-grown cells, but not in cells grown on benzoate or ethylbenzene. Another toluene-induced polypeptide band was visible at 45 kDa, as seen in *T. aromatica* and *D. toluolica*.

Table 2 Initial rates of ethylbenzene conversion into products [pmol min^{-1} (mg protein)$^{-1}$] in extracts of toluene- and ethylbenzene-grown cells of denitrifying strain EbN1 (*nd* not determined)

Supplements added (2 mM each)	Toluene-grown cells [a]	Ethylbenzene-grown cells
None	nd	< 1
Nitrate	< 1	2.3
Nitrate + bicarbonate	nd	12.5
Nitrate + bicarbonate + ATP + CoASH	< 1	12

[a] Note that these assays also contained fumarate

Ethylbenzene degradation by denitrifying strain EbN1

Extracts of strain EbN1 grown on ethylbenzene or toluene were tested for conversion of [*methylene*-^{14}C]-ethylbenzene. We found that extracts from ethylbenzene-grown cells converted labeled ethylbenzene to nonvolatile products at a rate of up to 10–30 pmol min^{-1} (mg protein)$^{-1}$ (Table 2). The products formed in extracts supplied with various supplements were analysed by TLC. In the absence of added supplements, the reaction rate was below the detection limit, but accumulation of some 1-phenylethanol was nevertheless recorded after more than 3 h of incubation. No further products were detected, indicating that 1-phenylethanol was not metabolised further in the absence of an electron acceptor (data not shown). In all assays supplied with nitrate as electron acceptor, labeled 1-phenylethanol and acetophenone were early products, as predicted from the proposed pathway of ethylbenzene degradation (Fig. 4). No labeled product comigrated with 2-phenylethanol, the other possible oxidation product of ethylbenzene.

The next predicted reactions in ethylbenzene metabolism are carboxylation of acetophenone, activation of the resulting β-oxo acid to a CoA-thioester, and thiolytic cleavage to benzoyl-CoA and acetyl-CoA (Fig. 1). Therefore, we investigated whether supplementation of extracts with bicarbonate, ATP and CoASH changed the product pattern produced from labeled ethylbenzene. The measured reaction rate of ethylbenzene oxidation increased approximately fivefold in the presence of bicarbonate and remained at that level when ATP and CoASH were added (Table 2). However, 1-phenylethanol and acetophenone were still the only products produced from ethylbenzene under these conditions (Fig. 4). Traces of labeled CoA-thioesters and some unidentified polar products were detected only after long-term incubation (> 12 h) in the presence of nitrate, bicarbonate, ATP and CoASH (Fig. 4). We do not know at present whether these trace products are physiologically relevant. Formation of 1-phenylethanol and acetophenone, plus a putative carboxylation product, from ethylbenzene has previously been reported from cell suspension experiments with the denitrifying, ethylbenzene-degrading strain EB1 (Ball et al. 1996).

The rate of conversion of [*methylene*-^{14}C]-ethylbenzene to nonvolatile products in extracts of toluene-grown cells of strain EbN1 was below the detection limit (Table 2). Still, low amounts of products were detected after overnight incubation. TLC analysis showed that two labeled products that comigrate with 1-phenylethanol and acetophenone (Fig. 4) were formed. This observation may be explained by the presence of low levels of ethylbenzene catabolic enzymes even in toluene-grown cells. This interpretation was reasserted by the lack of product formation from [^{14}C]-ethylbenzene in extracts of toluene-grown *T. aromatica*, which is unable to metabolise ethylbenzene (data not shown).

SDS-PAGE of cell extracts of ethylbenzene-grown cells of strain EbN1 revealed that a set of polypeptides was strongly induced after growth on ethylbenzene; these polypeptides were not present in benzoate- and toluene-grown cells. No polypeptides comigrating with the α/α′-subunits of benzylsuccinate synthase were detected in ethylbenzene-grown cells (Fig. 3). Because of the involvement of a carboxylation reaction in the proposed degradation pathway of ethylbenzene, we searched for ethylbenzene-induced biotinylated polypeptides. Three constitutive polypeptides of 16, 19 and 75 kDa carrying covalently bound biotin were identified in *T. aromatica* and in strain EbN1, but none was specifically induced in ethylbenzene-grown cells (data not shown).

Discussion

Anaerobic degradation of toluene appears to be generally initiated by addition of fumarate to the methyl group in bacteria of different physiological and phylogenetic groups. This has been verified in this communication for denitrifying toluene-degrading bacteria (*T. aromatica* and strain EbN1), which belong to the β-subgroup of Proteobacteria, and for a sulfate-reducing bacterium (*D. toluolica*) of the δ-subclass. This is most remarkable given the large difference in energy yield from toluene oxidation coupled to denitrification ($\Delta G^{\circ\prime}$ = –3529.6 kJ mol^{-1}) and sulfate reduction ($\Delta G^{\circ\prime}$ = –179.4 kJ mol^{-1}). However, benzylsuccinate formation appears to be an ideal initiation reaction for toluene metabolism under energy-limited conditions. It is exergonic ($\Delta G^{\circ\prime}$ = ca. –40 kJ mol^{-1}) and therefore only requires an appropriate catalyst to proceed. The enzyme catalysing this reaction, benzylsuccinate synthase, can be identified as a major toluene-induced protein on SDS-polyacrylamide gels and appears to be common to these bacteria. Conservation of the typical double-band pattern suggests strongly that the enzyme contains a glycyl radical in all analysed species. Although toluene-degrading ferric iron reducers and the fermentative bacteria present in toluene-degrading methanogenic consortia have not been analysed, they most likely utilise the same pathway as the related sulfate-reducing bacteria. The further steps of benzylsuccinate oxidation to benzoyl-CoA in *T. aromatica* resemble β-oxidation of fatty acids (Biegert et al. 1996; C. Leutwein, B. Leuthner and J. Heider, unpublished work) and are probably also common for all of these organisms.

Further metabolism of benzoyl-CoA, as established for denitrifying and photosynthetic bacteria, involves the reduction of the aromatic ring. This reaction requires the input of two ATP for the two-electron reduction of benzoyl-CoA to cyclohexa-1,5-diene-1-carboxy-CoA (Boll and Fuchs 1997). Energy-limited bacteria such as sulfate-reducers must be expected to accomplish benzoyl-CoA degradation differently because they cannot afford the required ATP investment of this enzyme. In accordance, the "denitrifying type" of benzoyl-CoA reductase was not detected in extracts of *D. toluolica* (R. Rabus and J. Heider, unpublished work).

Anaerobic metabolism of ethylbenzene by the denitrifying strain EbN1 proceeded via a pathway different from that of toluene degradation. We detected the formation of 1-phenylethanol and acetophenone from ethylbenzene in cell extracts. No further degradation products were obtained under the conditions used, indicating that the carboxylation reaction of acetophenone may need additional cofactors or cosubstrates to proceed in vitro. Still, the presented results verify the postulated pathway of ethylbenzene degradation, which has been deduced from growth experiments, simultaneous adaptation, and substrate conversion studies with whole cells (Rabus 1995; Rabus and Widdel 1995 a; Ball et al. 1996). The experiments reported here should also be a first step for purification and characterisation of enzymes involved in ethylbenzene metabolism. The distinctness of the metabolic pathway for toluene and ethylbenzene degradation in strain EbN1 is reflected by the polypeptide patterns. A number of proteins are highly induced in ethylbenzene-grown cells, but they are all different from those induced upon growth on toluene. The inducibility of these two pathways is further supported by studies with cell suspensions of ethylbenzene-grown cells of strain EbN1. These oxidised ethylbenzene immediately, whereas they required a pronounced adaptation period to obtain the capacity to degrade toluene (Rabus 1995).

Both initial reactions reported in this communication proceed at very low rates that correspond to only 0.1–1% of the rates required for the observed growth rates. A possible explanation in the case of toluene metabolism may be the extreme sensitivity of benzylsuccinate synthase to oxygen inactivation or to other agents (Leuthner et al. 1998). The strong induction of benzylsuccinate synthase synthesis in toluene-grown cells (Leuthner et al. 1998) and the more than 20-fold higher reaction rates in experiments with permeabilised cells (Beller and Spormann 1997 a, b) indicate that benzylsuccinate formation is indeed physiologically significant. In the case of ethylbenzene metabolism we do not know any possible reasons for the slow reaction rates. However, it seems to be the first step that is the most difficult. As shown previously, strain EbN1 utilises 1-phenylethanol and acetophenone, but not ethylbenzene, as growth substrates during aerobic growth conditions (Rabus and Widdel 1995 a).

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References

Altenschmidt U, Fuchs G (1991) Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication of toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch Microbiol 156 : 152–158

Ausubel FM et al. (1987) Current protocols in molecular biology. Wiley, New York

Ball HD, Johnson HA, Reinhard M, Spormann AM (1996) Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J Bacteriol 178 : 5755–5761

Beller HR, Spormann AM (1997 a) Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J Bacteriol 179 : 670–676

Beller HR, Spormann AM (1997 b) Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. Appl Environ Microbiol 63 : 3729–3731

Beller HR, Spormann AM, Sharma PK, Cole JR, Reinhard M (1996) Isolation and characterisation of a novel toluene-degrading sulfate-reducing bacterium. Appl Environ Microbiol 62 : 1188–1196

Biegert T, Fuchs G, Heider J (1996) Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur J Biochem 238 : 661–668

Boll M, Fuchs G (1995) Benzoyl-coenzyme A reductase (dearomatising), a key enzyme of anaerobic aromatic metabolism: ATP dependence of the reaction, purification and some properties of the enzyme from *Thauera aromatica* strain K172. Eur J Biochem 234 : 921–933

Boll M, Fuchs G (1997) Benzoyl-coenzyme A reductase (dearomatising), a key enzyme of anaerobic aromatic metabolism: a study of adenosinetriphosphatase activity, ATP stoichiometry of the reaction and EPR properties of the enzyme. Eur J Biochem 244 : 840–851

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72 : 248–254

Dolfing J, Zeyer P, Binder-Eicher P, Schwarzenbach RP (1990) Isolation and characterisation of a bacterium that mineralises toluene in the absence of molecular oxygen. Arch Microbiol 154 : 336–341

Evans PJ, Mang DT, Kim KS, Young LY (1991) Anaerobic degradation of toluene by a denitrifying bacterium. Appl Environ Microbiol 57 : 1139–1145

Fries MR, Zhou J, Chee-Sanford J, Tiedje JM (1994) Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl Environ Microbiol 60 : 2802–2810

Heider J, Fuchs G (1997) Microbial anaerobic aromatic metabolism. Anaerobe 3 : 1–22

Heider J et al. (1998) Differential induction of enzymes involved in anaerobic metabolism of aromatic compounds in the denitrifying bacterium *Thauera aromatica*. Arch Microbiol 170 : 120–131

Laemmli UK (1970) Cleavage of the structural proteins during the assembly of the head of the bacteriophage T4. Nature 227 : 680–685

Leuthner B et al. (1998) Biochemical and genetic characterisation of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl-radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol Microbiol 28 : 615–628

Lovley DR, Lonergan DJ (1990) Anaerobic oxidation of toluene, phenol and *p*-cresol by the dissimilatory iron-reducing organism GS15. Appl Environ Microbiol 56 : 1858–1864

Rabus R (1995) Anaerober Abbau von Alkylbenzolen und Rohöl durch nitratreduzierende und sulfatreduzierende Bakterien. PhD Thesis, Universität Bremen, Germany

Rabus R, Widdel F (1995a) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol 163:96–103

Rabus R, Widdel F (1995b) Conversion studies with substrate analogues of toluene in a sulfate-reducing bacterium, strain Tol2. Arch Microbiol 164:448–451

Rabus R, Nordhaus R, Ludwig W, Widdel F (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl Environ Microbiol 59:1444–1451

Rabus R, Fukui M, Wilkes H, Widdel F (1996) Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl Environ Microbiol 62:3605–3613

Schocher RJ, Seyfried B, Vazquez F, Zeyer J (1991) Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch Microbiol 157:7–12