Arch Microbiol (1998) 170 : 120–131

ORIGINAL PAPER

Johann Heider · Matthias Boll · Klaus Breese
Sabine Breinig · Christa Ebenau-Jehle · Ulrich Feil
Nasser Gad'on · Diana Laempe · Birgitta Leuthner
Magdy El-Said Mohamed · Sabine Schneider
Gerhard Burchhardt · Georg Fuchs

Differential induction of enzymes involved in anaerobic metabolism of aromatic compounds in the denitrifying bacterium *Thauera aromatica*

Received: 22 December 1997 / Accepted: 12 May 1998

Abstract Differential induction of enzymes involved in anaerobic metabolism of aromatic substrates was studied in the denitrifying bacterium *Thauera aromatica*. This metabolism is divided into (1) peripheral reactions transforming the aromatic growth substrates to the common intermediate benzoyl-CoA, (2) the central benzoyl-CoA pathway comprising ring-reduction of benzoyl-CoA and subsequent β-oxidation to 3-hydroxypimelyl-CoA, and (3) the pathway of β-oxidation of 3-hydroxypimelyl-CoA to three acetyl-CoA and CO_2. Regulation was studied by three methods.

1. Determination of protein patterns of cells grown on different substrates. This revealed several strongly substrate-induced polypeptides that were missing in cells grown on benzoate or other intermediates of the respective metabolic pathways.

2. Measurement of activities of known enzymes involved in this metabolism in cells grown on different substrates. The enzyme pattern found is consistent with the regulatory pattern deduced from simultaneous adaptation of cells to utilisation of other aromatic substrates.

3. Immunological detection of catabolic enzymes in cells grown on different substrates. Benzoate-CoA ligase and 4-hydroxybenzoate-CoA ligase were detected only in cells yielding the respective enzyme activity. However, presence of the subunits of benzoyl-CoA reductase and 4-hydroxybenzoyl-CoA reductase was also recorded in some cell batches lacking enzyme activity. This possibly indicates an additional level of regulation on protein level for these two reductases.

Key words Anaerobic aromatic metabolism · Benzoyl-CoA reductase · Phenylphosphate carboxylase · 4-Hydroxybenzoyl-CoA reductase · 2-Aminobenzoate · Phenylalanine · Phenylacetyl-CoA · Phenylglyoxylate · Toluene · CoA ligase

J. Heider (✉) · M. Boll · K. Breese · S. Breinig · C. Ebenau-Jehle
U. Feil · N. Gad'on · D. Laempe · B. Leuthner
M. El-Said Mohamed[1] · S. Schneider · G. Fuchs
Institut für Biologie II, Universität Freiburg, Schänzlestrasse 1,
D-79 104 Freiburg, Germany
e-mail heiderj@ruf.uni-freiburg.de
Tel. +49-761-203-2774; Fax +49-761-203-2626

G. Burchhardt
Institut für Genetik und Biochemie, Universität Greifswald,
D-17 487 Greifswald, Germany

Permanent address:
[1] Department of Botany, University of Cairo, Giza, Egypt

Introduction

Aromatic compounds can be metabolised by microorganisms both aerobically and anaerobically. In the presence of air, oxygen is always used as a cosubstrate to attack the chemically inert aromatic ring [last reviewed by Harwood and Parales (1996)]. Bacteria capable of degrading aromatic compounds anaerobically obviously need different pathways to attack the stable ring systems. These have in common a reductive dearomatising step of the aromatic ring (Heider and Fuchs 1997).

Many studies of anoxic aromatic metabolism have been performed with the denitrifying bacterium *Thauera aromatica*, which is capable of using aromatic compounds as sole carbon sources for both aerobic and anaerobic growth. Its aromatic substrate spectrum is different during aerobic and anaerobic growth, and it uses different pathways in aerobic and anaerobic degradation of aromatic compounds (Anders et al. 1995). In the absence of oxygen, *T. aromatica* first transforms all aromatic substrates studied to date to benzoyl-CoA, the central intermediate of its anaerobic aromate degradation pathway. These reactions constitute the peripheral aromatic metabolism of this species (Fig. 1). The aromatic ring of benzoyl-CoA is then reduced by benzoyl-CoA reductase to the non-aromatic product cyclohexa-1,5-diene-1-carboxyl-CoA; this first intermediate is oxidised to 3-hydroxypimelyl-CoA via 2,3-unsaturated pimelyl-CoA as the first open-chain product (D. Laempe and G. Fuchs, unpublished work). This

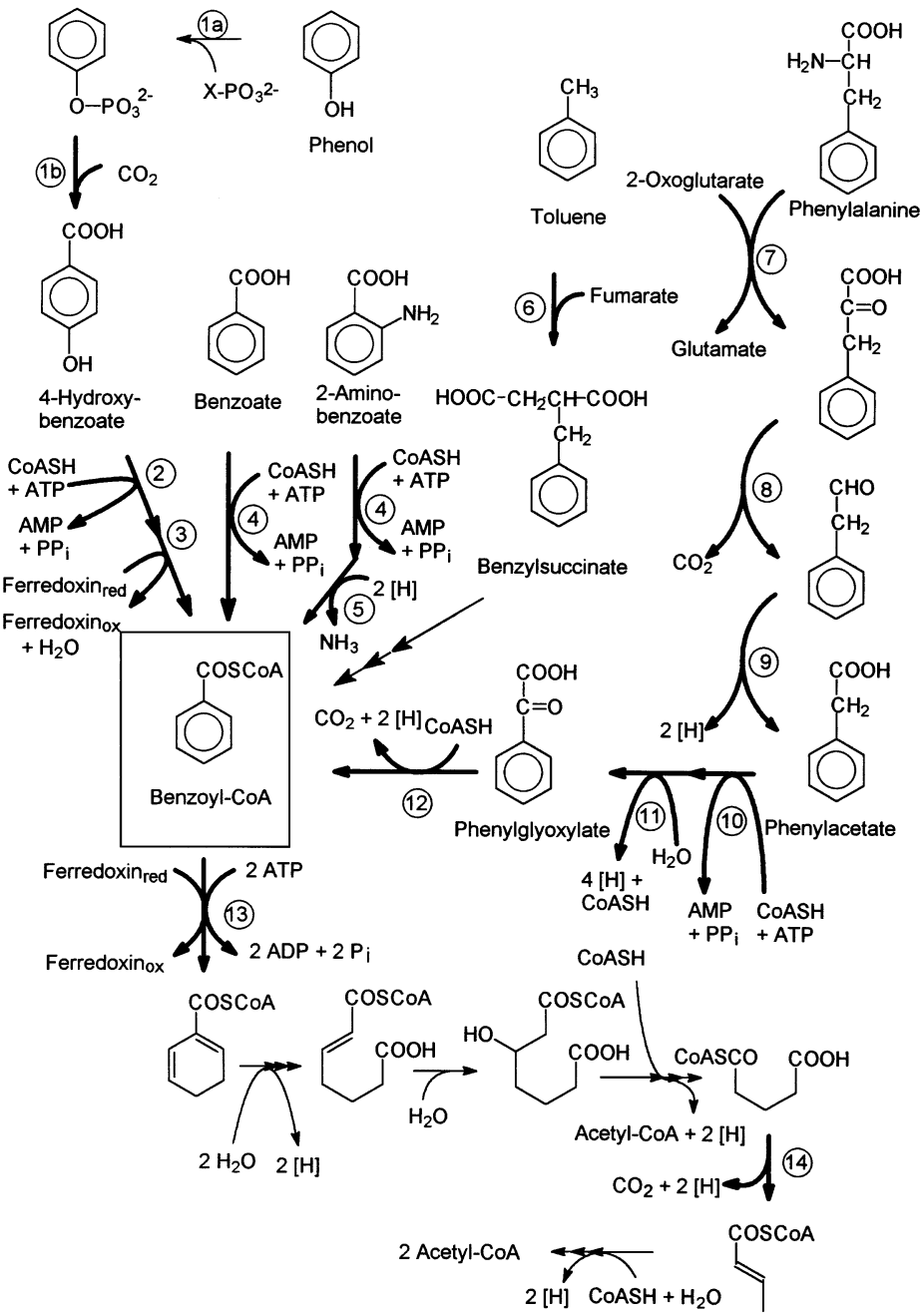

Fig. 1 Overview of the anaerobic metabolism of aromatic compounds in *Thauera aromatica.* Enzyme reactions reported in this communication are shown with *thick arrows*. The enzymes catalysing these reactions are: *1* Phenol carboxylating enzyme system with phenol phosphorylase *(1a)* and phenylphosphate carboxylase *(1b)*; *2* 4-hydroxybenzoate-CoA ligase, *3* 4-hydroxybenzoyl-CoA reductase, *4* benzoate-CoA ligase, *5* 2-aminobenzoyl-CoA reducing enzyme, *6* benzylsuccinate synthase, *7* Phenylalanine-2-oxoglutarate aminotransferase, *8* phenylpyruvate decarboxylase, *9* phenylacetaldehyde dehydrogenase, *10* phenylacetate-CoA ligase, *11* phenylacetyl-CoA-oxidising enzyme system, *12* phenylglyoxylate-acceptor oxidoreductase, *13* benzoyl-CoA reductase, and *14* glutaryl-CoA dehydrogenase

common pathway is referred to as the central benzoyl-CoA pathway. Finally, 3-hydroxypimelyl-CoA is oxidised to three acetyl-CoA and CO_2 by a modified β-oxidation pathway. This terminal pathway is not specific for aromatic compounds, but is shared with C_7 and other odd-numbered dicarboxylic acids [for a review, see Heider and Fuchs (1997)].

The peripheral pathways leading to benzoyl-CoA have been elucidated for a number of substrates. A characteristic feature is the use of coenzyme-A thioesters of aromatic acids, which are formed from aromatic acids by more or less specific CoA ligases [AMP-forming; see Heider and Fuchs (1997)]. Synthesis of the specific CoA ligases is expected to be induced during anaerobic growth on the respective aromatic acids. Moreover, a parallel set of "aerobic" isoenzymes is specifically induced upon aerobic growth on some aromatic substrates (Altenschmidt et al. 1991; M. Mohamed and G. Fuchs, unpublished work). Other reactions involved in peripheral metabolism of aromatic compounds include reductive dehydroxylation and deamination, methyl- and methylene-group oxidation, alcohol and aldehyde oxidation, carboxylation, and oxidative and nonoxidative decarboxylation [reviewed in Heider and Fuchs (1997)]. In the course of our studies on the biochemistry of anaerobic aromatic substrate degradation, we observed that many of the enzyme activities catalysing peripheral reactions are present only after anaerobic growth on the respective substrate. These enzymes appear

to be regulated in response to oxygen and substrate availability, and additional catabolite regulation effects may occur.

This work is an extension of a previous investigation on the presence of some enzyme activities involved in anaerobic degradation of aromatic compounds in differently grown cells (Dangel et al. 1991). It is intended to provide a deeper insight into the basic regulation patterns of degradation pathways in *T. aromatica* and to serve as a foundation for studying their regulation on the molecular level. We have analysed enzyme induction in response to oxic/anoxic conditions and to the presence of different aromatic and nonaromatic substrates under denitrifying conditions. We investigated specific activities of several key enzymes involved in the degradation of aromatic compounds and differences in whole-cell protein patterns in cells grown on 11 different compounds as the sole carbon and energy source; finally, the amount of some enzymes was monitored by immunoblot analysis.

Material and methods

Bacterial growth and preparation of cell extracts

T. aromatica was grown aerobically or anaerobically in mineral salts medium at 28°C with different organic substrates (1–5 mM) as the carbon and energy source and with nitrate (10 mM initial concentration) as the terminal electron acceptor. The different substrates used are indicated in Results. The bacteria were subcultured at least two times on a given substrate before large-scale cultures were grown in a 10- or a 200-l fermenter, depending on the substrate. Further details on cell growth and harvesting were as described previously (Dangel et al. 1991). Cell extracts were prepared at 4° C under strictly anoxic conditions. Frozen cells (2–10 g, wet mass) were resuspended in 100 mM triethanolamine hydrochloride/NaOH buffer (pH 8.0) containing 2 mM $MgCl_2$, 10% glycerol and 0.1 mg DNase/ml and were passed through a French press cell at 137 MPa. Cell lysates were centrifuged at $100{,}000 \times g$ for 90 min. For measuring the membrane-bound phenylacetyl-CoA-oxidising enzyme complex, membranes were prepared by centrifuging $20{,}000 \times g$ supernatant at $100{,}000 \times g$ for 90 min; the pellet was washed and resuspended in 100 mM triethanolamine hydrochloride/NaOH buffer (pH 8.0) containing 2 mM $MgCl_2$ and 10% glycerol.

Electrophoretic methods

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Separated polypeptides were either directly transferred to nitrocellulose membranes or stained with Coomassie blue. Molecular mass standards were phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), lactate dehydrogenase (34 kDa), carboanhydrase (29 kDa) and lysozyme (14 kDa).

Protein was separated by two-dimensional gel electrophoresis according to O'Farell (1975). The first dimension (isoelectric focusing) was performed in tube gels (6.6% polyacrylamide) using an ampholine mixture of 3.4% (pH 5–7) and 0.9% (pH 3–10) in the gels and lysis buffers. Extract (300 µg) was applied to each tube, and the proteins were focused for 11500 Vh. The gels were then removed from the tubes and incubated in denaturing buffer [60 mM Tris-HCl (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.005% bromophenolblue]. The proteins were separated in the second dimension by SDS-polyacrylamide gel electrophoresis as described above.

Immunoblotting

Cell extracts were separated by SDS-gel electrophoresis and were blotted on nitrocellulose sheets (Schleicher & Schüll, Dassel, Germany) using a Multiphor system (Pharmacia). Proteins were immunologically detected by luminescence using the ECL system (Amersham) or by the immunostaining procedure according to Rao et al. (1983).

Enzyme assays

Benzoyl-CoA reductase

The assay for benzoyl-CoA reductase was performed essentially as described by Koch and Fuchs (1992). The reduction of [*phenyl*-^{14}C]-benzoyl-CoA to nonaromatic products was followed by HPLC analysis. The assay was performed under an N_2 atmosphere at 30° C in stoppered tubes containing 800 µl of 150 mM Mops/KOH (pH 7.3), 10 mM $MgCl_2$, 5 mM ATP, 0.2 mM [*phenyl*-^{14}C]-benzoyl-CoA (specific radioactivity, 2.0 GBq $mmol^{-1}$) and 5 mM Ti(III)citrate. The reaction was started by adding 50 µl extract; samples of 100 µl were taken at 3-min intervals, mixed with 10 µl 10% formic acid, and centrifuged. The supernatants (20 µl) were applied to an RP C-18 Lichrosphere HPLC column [Merck; eluent, 11% acetonitrile in 50 mM potassium phosphate (pH 6.7)]. ^{14}C-labeled CoA-thioesters were quantitated by monitoring eluting radioactivity with a radioactivity detector (Ramona; Raytest, Straubenhardt, Germany).

Phenyl phosphate carboxylase

The assay mixture for CO_2 isotope exchange (1 ml) contained 20 mM imidazole-Cl (pH 6.5), 0.5 mM $MnCl_2$, 20 mM KCl, 2 mM 4-hydroxybenzoate, 50 mM $NaHCO_3$, 5.25 kBq [^{14}C]-Na_2CO_3 (specific radioactivity, 2.0 GBq/mmol) and 25 µl extract (protein concentrations of 55–65 mg/ml). The components except for [^{14}C]-Na_2CO_3 were combined and preincubated for 5 min at 30°C; then the test was started by the addition of 5.25 kBq [^{14}C]-Na_2CO_3 (specific radioactivity, 2.0 GBq/mmol). Samples of 200 µl were taken after 4 min, mixed with 30 µl 3 M perchloric acid, and centrifuged for 10 min. Supernatants (200 µl) were mixed with 150 µl 10 M formic acid, and [^{14}C]-CO_2 was extruded by gassing with CO_2 for 15 min. Then 100 µl 1 M $KHCO_3$ was added, and CO_2 formed was extruded as before. Acid-stable radioactivity in the supernatants was measured by liquid scintillation counting (Lack and Fuchs 1992). The phenyl phosphate carboxylase assay was essentially identical, except that 4-hydroxybenzoate was omitted and the reaction was started by adding 2 mM phenyl phosphate as substrate (Lack and Fuchs 1992).

4-Hydroxybenzoyl-CoA reductase

The assay for 4-hydroxybenzoyl-CoA reductase was performed according to Brackmann and Fuchs (1993) with minor modifications. Crude extracts were precipitated with ammonium sulfate (60% saturation) to reduce unspecific oxidation of methyl viologen in the test. The precipitate was resuspended in the original volume of anoxic 20 mM triethanolamine-HCl buffer (pH 7.8) containing 200 mM KCl. The assay was performed in anoxic cuvettes containing 300 µl 50 mM potassium phosphate (pH 7.0), 2.5 mM $MgCl_2$, 1 mM methyl viologen, sodium dithionite to reduce the viologen to an A_{730} of ca. 1.0, and 10 µl of resuspended ammonium sulfate precipitate. The reaction was started by the addition of 0.2 mM 4-hydroxybenzoyl-CoA, and the decrease in A_{730} was monitored. An extinction coefficient at 730 nm of 2,400 M^{-1} cm^{-1} for reduced methyl viologen and a stoichiometry of 2 mol viologen oxidised per mol 4-hydroxybenzoyl-CoA reduced were assumed.

2-Aminobenzoyl-CoA reductase

The enzymatic reduction of 2-aminobenzoyl-CoA was studied under strictly anoxic conditions at 30°C. Assays were performed in stoppered glass cuvettes under an N_2 atmosphere. All components were added with gas-tight microliter syringes. The assay mixture (0.5 ml total volume) contained 150 mM Mops-KOH buffer (pH 7.2), 0.2 mM 2-aminobenzoyl-CoA, 4 mM $MgCl_2$, 4 mM ATP, 3 mM Ti-(III)citrate and 10 µl of cell extract. The reduction of 2-aminobenzoyl-CoA was monitored spectrophotometrically at 365 nm (ε = 5.5 $mM^{-1}cm^{-1}$). The reaction was started by adding Ti(III)citrate, and the decrease in A_{365} was recorded.

Benzylsuccinate synthase

The test for benzylsuccinate synthase activity was a modification of the procedure described in Biegert et al. (1996). Briefly, Na-fumarate was added to 200 µl anoxic cell extract (final concentration, 10 mM). The reaction was started by adding [*phenyl-*^{14}C]-toluene (specific radioactivity, 218 MBq/mmol; final concentration, 200 µM). Samples were taken after different times, and the reaction was stopped by adding 5% H_2SO_4 (v/v, final concentration). Precipitated protein was removed by centrifugation, and the supernatants were used for determining the conversion of [*phenyl-*^{14}C]-toluene to nonvolatile compounds by scintillation counting. Previous experiments have shown that benzylsuccinate is the only nonvolatile compound produced under these conditions.

Phenylalanine 2-oxoglutarate transaminase

This activity was detected in 100,000 × *g* extracts at 37°C as described by Schneider et al. (1997).

Phenylpyruvate decarboxylase

This activity was tested in 100,000 × *g* extracts at 37°C by a coupled enzyme assay (Schneider et al. 1997).

Phenylacetaldehyde dehydrogenase

This test was performed with 100,000 × *g* extracts at 37°C as described in Schneider et al. (1997).

Phenylacetyl-CoA acceptor oxidoreductase

The enzymatic α-oxidation of phenylacetyl-CoA to phenylglyoxylate was determined in resuspended membrane fractions at 30°C under anoxic conditions. The assay was performed in 50 mM potassium phosphate (pH 7.5) containing 0.25 mM dichlorophenolindophenol (DCPIP) as an artificial electron acceptor and 20 µl membrane fraction. The reaction was started by adding 0.1 mM phenylacetyl-CoA, and reduction of DCPIP was monitored spectrophotometrically at 546 nm (ε_{546} = 13,800 M^{-1} cm^{-1}). The specific activities given here relate to the protein concentrations of nonfractionated cell extracts.

Phenylglyoxylate acceptor oxidoreductase

This enzyme was measured according to Hirsch et al. (1998) as phenylglyoxylate- and CoA-dependent benzyl-viologen-reducing activity.

CoA ligases

All extracts were precipitated twice by ammonium sulfate (65% saturation) to decrease unspecific background activity and deplete inhibitory components of the extracts. Specific activities given are normalised to the protein concentrations of untreated extracts. Most CoA ligase activities were determined in a coupled spectrophotometric assay as described previously (Ziegler et al. 1989). The tests were started by the addition of 0.5 mM of the organic acids. 2-Aminobenzoate-CoA ligase activity was monitored directly utilising the absorption of 2-aminobenzoyl-CoA at 365 nm (ε = 5500 $M^{-1}cm^{-1}$), in 100 mM Tris-HCl (pH 7.8) containing 5 mM $MgCl_2$, 1 mM ATP, 0.4 mM CoA, 1 mM phosphoenolpyruvate, 9 nkat myokinase and 13 nkat pyruvate kinase. Myokinase was omitted in the assay of succinate-CoA ligase.

CoA transferase

Succinyl-CoA:glutarate-CoA transferase was measured in ammonium sulfate-precipitated extracts by a modified succinate-CoA ligase assay. The test was performed in 100 mM Tris-HCl pH 8.4 containing 5 mM $MgCl_2$, 0.4 mM NADH, 0.4 mM CoA, 1 mM ITP, 1 mM phosphoenolpyruvate, 17 nkat pyruvate kinase, 30 nkat lactate dehydrogenase, 3 nkat succinyl-CoA ligase and 0.05 mM succinate. When the limiting amount of succinate was completely converted to succinyl-CoA, 10 µl of extract was added, and the test was started by adding 0.5 mM glutarate.

Glutaryl-CoA dehydrogenase

Glutaryl-CoA dehydrogenase/decarboxylase was measured according to Härtel et al. (1993) with the ferricenium ion as an artificial electron acceptor. The test was performed at 30° C in 100 mM Tris-HCl (pH 7.5) containing 0.2 mM ferricenium-PF_6 and 2–8 ng of ammonium-sulfate-precipitated crude extracts (65% saturation) and was started by adding 0.2 mM glutaryl-CoA. The decrease in absorbance at 300 nm was followed; an extinction coefficient of 4,300 $M^{-1}cm^{-1}$ was assumed for the ferricenium ion.

Materials

Chemicals and media components were obtained from Merck, Roth, Difco, Fluka, Sigma or Serva. Gases were from Linde. Enzymes against which antibodies were raised were purified as described previously (Altenschmidt et al. 1991; Biegert et al. 1993; Brackmann and Fuchs 1993; Boll and Fuchs, 1995). Antibodies were raised in mice or rabbits in the Departments of Immunology of the Universities of Ulm and Greifswald. Immunochemicals were from Sigma or Amersham, radioactive compounds from Amersham or Biotrend/ARC. Ferricenium hexafluorophosphate was synthesised according to Lehman et al. (1990); CoA-thioesters of organic acids were synthesised from the anhydrides according to Schachter and Taggart (1976) or via succinimidyl esters according to Gross and Zenk (1966). *T. aromatica* K172 was isolated by Tschech and Fuchs (1987) and described by Anders et al. (1995).

Results

Protein patterns of extracts of *T. aromatica* grown on different substrates

Phenol-grown cells of *T. aromatica* contained large amounts of polypeptides of 67, 60, 58, 24, 17 and 12 kDa, which were lacking in cells grown on the next compound along the metabolic pathway, 4-hydroxybenzoate (Fig. 2, lanes 10 and 11). These were confirmed by 2D PAGE analysis (Fig. 3 C). Cells grown on phenol or on 4-hydroxybenzoate, as compared to benzoate-grown cells, contained further induced polypeptides of 75, 35 and 17 kDa,

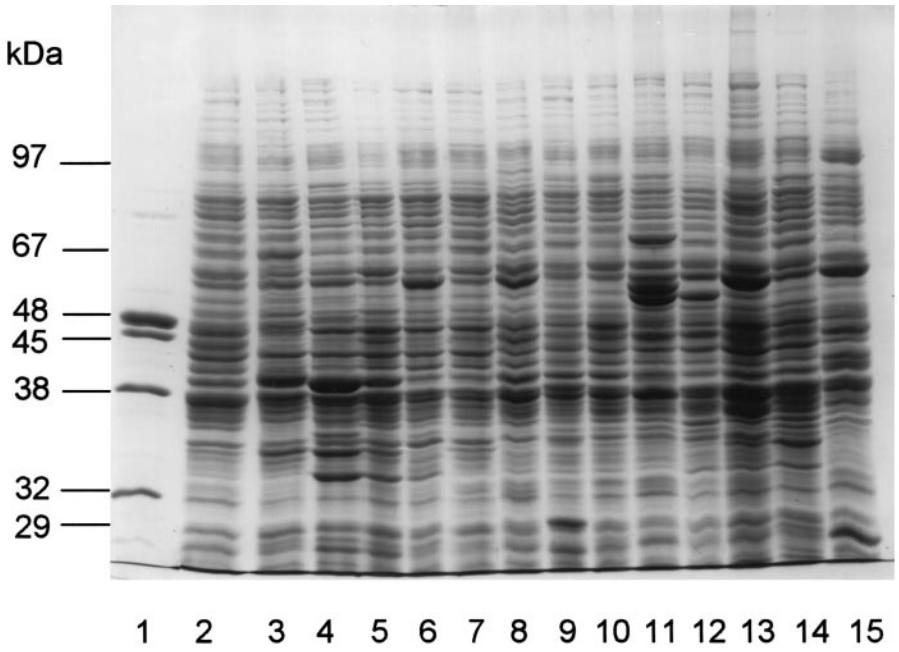

Fig. 2 SDS-PAGE of cell free extracts of *Thauera aromatica* grown on different substrates. Extracts were separated in a gel containing 10% (w/v) polyacrylamide and stained with Coomassie Blue. *Lanes 1* Purified benzoyl-CoA reductase, *2* cells grown anaerobically on benzoate, *3* cells grown aerobically on benzoate, *4–15* cells grown anaerobically on glutarate *(4)*, glutarate plus benzoate *(5)*, acetate *(6)*, acetate plus benzoate *(7)*, 2-aminobenzoate *(8)*, 3-hydroxybenzoate *(9)*, 4-hydroxybenzoate *(10)*, phenol *(11)*, phenylalanine *(12)*, phenylacetate *(13)*, phenylglyoxylate *(14)* and toluene *(15)*. Molecular masses of standard proteins and the subunits of benzoyl-CoA reductase are given along the *left margin*

as revealed by 2D PAGE (Fig. 3 C, D). These may correspond to the subunits of 4-hydroxybenzoyl-CoA reductase, as suggested by matching masses and apparent pI values of 5.3 for the α (pI_{calc} 5.8) and 7.7 for the β (pI_{calc} 8.2) subunits (Breese and Fuchs 1998). The two induced proteins of 17 kDa may be differently charged forms of the γ-subunit. Four major polypeptides present in benzoate-grown cells disappeared upon growth on phenol and 4-hydroxybenzoate (labeled by open triangles in Fig. 3 B).

Toluene-grown cells contained at least six toluene-induced polypeptides as compared to benzoate-grown cells: a doublet of 90/94 kDa and additional proteins of the molecular masses 60, 45 and 30 kDa (Fig. 2, lane 15; Fig. 3 A, B). The polypeptide doublet of 94 and 90 kDa was hardly detectable in 2D gels because these proteins did not focus sharply in the first dimension. It represents two forms (α and α′) of the large subunit of the first enzyme involved in toluene degradation, benzylsuccinate synthase (Leuthner et al. 1998). One of the further toluene-induced polypeptides represents a thiolase involved in a β-oxidation pathway from benzylsuccinate to benzoyl-CoA (B. Leuthner and J. Heider, unpublished work).

Cells grown on 3-hydroxybenzoate (Fig. 2, lane 9) and 2-aminobenzoate (Fig. 2, lane 8) contained clearly induced polypeptides that to date cannot be attributed to metabolic functions. The differences in protein patterns of cells grown on phenylalanine, phenylacetate and phenylglyoxylate (Fig. 2, lanes 12–14) were less pronounced; to date we cannot correlate these to different enzyme activities. Anaerobic growth on glutarate elicited the synthesis of large amounts of a 39-kDa polypeptide and some additional polypeptides (at 60, 47, 35 and 33 kDa). A similar situation was recorded for growth on acetate. Acetate as the sole carbon source induced synthesis of a 60-kDa polypeptide and several others (Fig. 2, lane 6). Some of the acetate- and glutarate-induced polypeptides appear to be identical, judging from the similar patterns. Upon growth on both glutarate and benzoate or on a mixture of acetate and benzoate, mixed polypeptide patterns were obtained (Fig. 2, lanes 5 and 7). We also analysed cells grown aerobically on benzoate: as expected, these showed a very different protein composition, which reflected their different physiological status (Fig. 2, lane 3).

Activities of catabolic enzymes involved in degradation of aromatic compounds

Enzymes of peripheral pathways of anaerobic degradation of aromatic compounds

Benzoate degradation by *T. aromatica* is initiated by benzoate-CoA ligase. Activity of this enzyme was detected in all extracts tested (Table 1). Significantly decreased levels were recorded in cells grown anaerobically on phenylglyoxylate, 2-aminobenzoate, 3-hydroxybenzoate and the non-aromatic substrates acetate and glutarate. Cells grown aerobically on benzoate also contained benzoate-CoA ligase activity (Table 1). This indicates that aerobic benzoate degradation in *T. aromatica* involves activation to the CoA-thioester as described in the related species *Azoarcus evansii* (Altenschmidt et al. 1993).

In anaerobic phenol degradation, phenyl phosphate becomes carboxylated to 4-hydroxybenzoate. The enzyme catalysing this reaction, phenyl phosphate carboxylase, was measured either by net carboxylation assays of phenyl phosphate or by carboxyl exchange assays between 4-hydroxybenzoate and free CO_2. Activity was exclusively detected in phenol-grown cells; cells grown on the next intermediate of the degradation pathway, 4-hydroxybenzoate, or on other aromatic substrates did not contain this enzyme (Table 2).

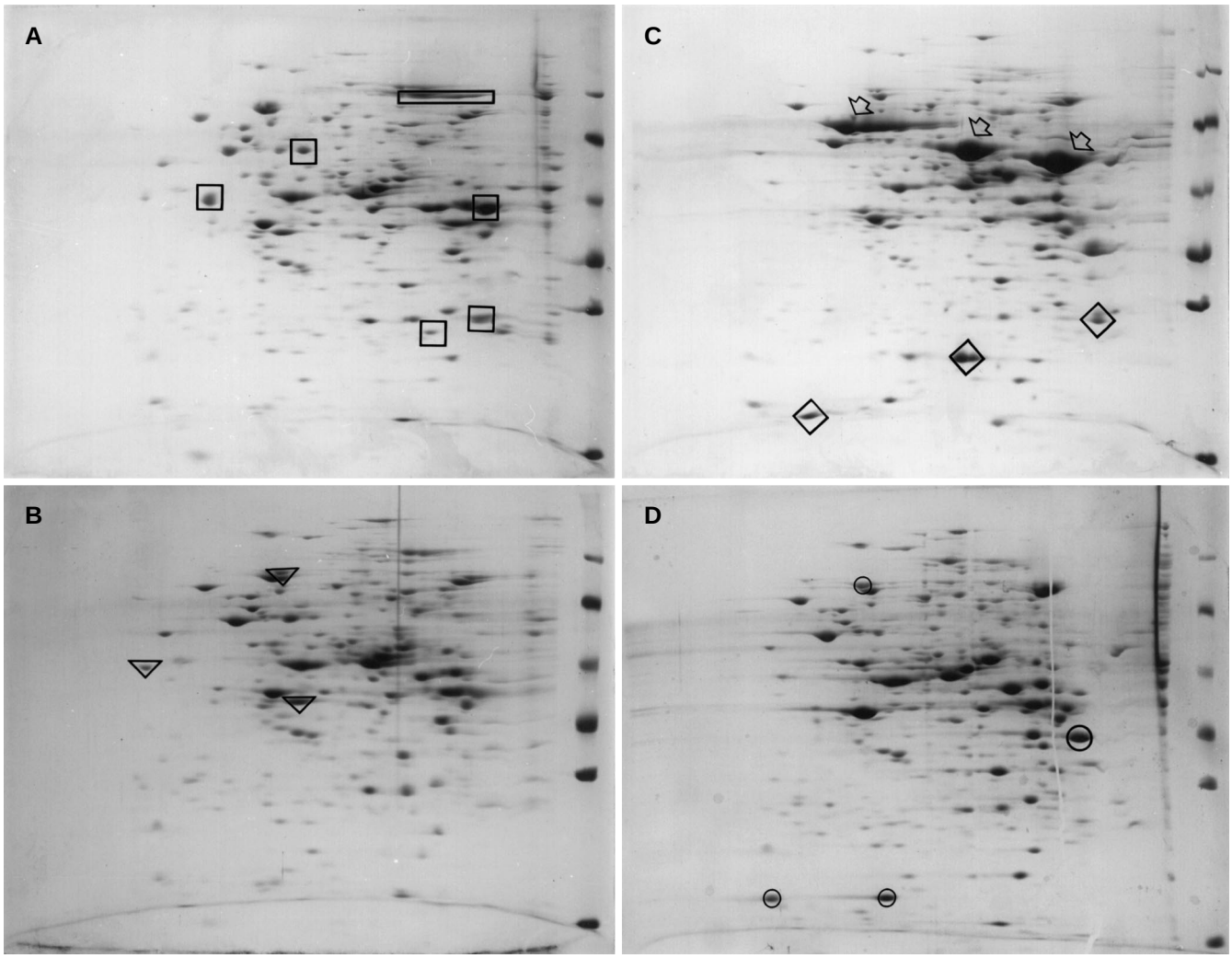

Fig. 3 Two-dimensional gel electrophoresis of cell extracts of *Thauera aromatica* grown anaerobically on **A** toluene, **B** benzoate, **C** phenol and **D** 4-hydroxybenzoate. The first dimension extended over a pH range from 4.5 *(left)* to 8.0 *(right)*.The pI values of phenol-induced proteins were determined from a 2D gel with an immobilised pH gradient in the first dimension (pH 3–10; Pharmacia) and were used as markers to determine further pI values in overlaid 2D gels. Molecular mass markers for the second dimension (11.5% polyacrylamide gel) are on the *right margins*: phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), lactate dehydrogenase (34 kDa), carboanhydrase (29 kDa) and lysozyme (14 kDa). **A** Toluene-induced polypeptides lacking in benzoate-grown cells are labeled by *squares*. Coordinates [pI/kDa]: 4.7/45, 5.6/60, 6.0–7.4/90–94, 6.3/26, 7.5/45, 7.5/29. **B** The *triangles* indicate polypeptides present in benzoate- and toluene-grown cells, but not in phenol- and 4-hydroxybenzoate-grown cells. Coordinates [pI/kDa]: 4.5/45, 5.3/75 (two spots), 5.4/38. **C** Phenol-induced polypeptides lacking in 4-hydroxybenzoate-grown cells are labeled with *arrows* (major spots) or *diamonds* (minor spots). Coordinates [pI/kDa]: 4.7/12, 4.8–5.2/67, 5.8/17, 5.9/60, 6.6/58, 7.5/24. **D** Polypeptides present in 4-hydroxybenzoate- and phenol-grown cells, but not in benzoate-grown cells, are indicated by *circles*. Coordinates [pI/kDa]: 4.7/17, 5.3/75, 5.7/17, 7.7/35

4-Hydroxybenzoate is degraded by conversion to the CoA-thioester and reductive dehydroxylation to benzoyl-CoA; these reactions are catalysed by 4-hydroxybenzoate-CoA ligase and 4-hydroxybenzoyl-CoA reductase. As expected, both enzyme activities were induced in cells grown on 4-hydroxybenzoate and phenol, which is degraded via 4-hydroxybenzoate (Tables1, 2). *T. aromatica* grown on 3-hydroxybenzoate contained a CoA ligase acting on 3-hydroxybenzoate (Table 1). This must be a novel isoenzyme since 4-hydroxybenzoate-CoA ligase does not act on other isomers (Biegert et al. 1993). Thus, 3-hydroxybenzoate degradation is apparently also initiated by formation of the CoA thioester. No activity of 4-hydroxybenzoyl-CoA reductase was detected in 3-hydroxybenzoate-grown cells, and no enzyme catalysing reductive dehydroxylation of 3-hydroxybenzoyl-CoA could be detected so far.

Anaerobic degradation of 2-aminobenzoate by *T. aromatica* affords conversion to the CoA-thioester, which is catalysed by benzoate CoA ligase. *T. aromatica* does not synthesise a separate CoA ligase isoenzyme for 2-aminobenzoate (U. Feil and G. Fuchs, unpublished work). The next enzyme in the proposed degradation pathway is 2-aminobenzoyl-CoA reductase (deaminating), which catalyses the reduction of 2-aminobenzoyl-CoA to benzoyl-CoA (Lochmeyer et al. 1992). The assay for this enzyme yielded the highest values in 2-aminobenzoate-grown cells, but high background levels were recorded in other

Table 1 Activities of CoA ligases (AMP-forming) acting on various aromatic and nonaromatic acids and of related enzymes in *Thauera aromatica* cells grown under different conditions [nmol min^{-1} (mg protein)$^{-1}$]. The values given are the average of at least two parallel measurements with less than 10% standard deviation. (*nd* not determined)

Growth substrate	Benzoate-CoA ligase	2-Amino-benzoate-CoA ligase	Phenyl-acetate-CoA ligase	3-Hydroxy benzoate-CoA ligase	4-Hydroxy benzoate-CoA ligase	Acetate-CoA ligase	Succinyl-CoA gluta-rate-CoA transferase	Succinate-CoA ligase (ADP-forming)	Thio-esterase	Glutaryl-CoA dehydro-genase
Benzoate	115	25	< 1	< 1	< 1	129	7	236	2	345
Phenol	109	32	< 1	2	30	91	< 1	169	nd	75
4-Hydroxybenzoate	78	19	9	6	51	65	10	285	nd	318
3-Hydroxybenzoate	60	16	< 1	62	56	18	10	257	3	382
2-Aminobenzoate	45	18	7	< 1	< 1	242	3	90	0.6	136
Toluene	126	43	3	6	5	109	9	133	nd	136
Phenylalanine	194	50	52	2	< 1	120	8	116	nd	233
Phenylacetate	114	37	88	< 1	< 1	93	22	322	nd	283
Phenylglyoxylate	33	8	3	< 1	< 1	106	15	236	nd	332
Acetate	58	14	< 1	< 1	< 1	424	12	228	2	88
Glutarate	36	11	2	< 1	< 1	92	48	186	1	873
Benzoate + Oxygen	89	18	5	23	< 1	192	7	124	5	80

Table 2 Activities of other enzymes involved in the degradation of aromatic compounds in *Thauera aromatica* grown under various conditions [nmol min^{-1} (mg protein)$^{-1}$]. The values given are the average of at least two measurements with less than 15% standard deviation. Activity values of phenyl phosphate carboxylase are from the carboxyl exchange assay with hydroxybenzoate, those obtained from carboxylation of phenyl phosphate are given in brackets. (*nd* not determined)

Growth substrate	Ben-zoyl-CoA reduc-tase	Phenyl-phosphate-carboxylase	4-Hydroxy-benzoyl-CoA reductase	Phenyl-alanine: 2-oxo-glutarate trans-aminase	Phenyl-pyruvate decarb-oxylase	Phenyl-acetalde-hyde de-hydro-genase	Phenyl-acetyl-CoA– oxi-dising enzyme	Phenyl-glyoxy-late oxido-reduc-tase	Benzyl-succi-nate synthase	2-Amino-benzoyl-CoA reducing enzyme
Benzoate	20	< 10 (< 0.1)	< 1	4	4	9	0.7	2	< 0.01	7
Phenol	22	383 (10)	26	nd	nd	nd	nd	2	< 0.01	8
4-Hydroxybenzoate	15	< 10 (< 0.1)	43	42	7	6	nd	2	nd	12
3-Hydroxybenzoate	2	< 10 (< 0.1)	< 1	nd	nd	nd	nd	2	nd	0
2-Aminobenzoate	35	nd	nd	nd	nd	nd	nd	1	< 0.01	16
Toluene	20	nd	nd	nd	nd	nd	nd	5	0.2	nd
Phenylalanine	14	nd	nd	558	169	215	16	45	nd	nd
Phenylacetate	13	nd	nd	15	12	23	11	48	nd	nd
Phenylglyoxylate	15	nd	nd	90	7	22	2	21	nd	nd
Acetate	< 1	nd	nd	nd	nd	nd	nd	< 0.1	nd	nd
Glutarate	< 1	nd	nd	nd	nd	nd	nd	3	nd	nd
Benzoate + oxygen	< 1	nd	nd	nd	nd	nd	nd	< 0.1	nd	nd
Phenylacetate + oxygen	nd	nd	nd	nd	nd	nd	< 0.1	nd	nd	nd

extracts (Table 2). This is due to the capacity of benzoyl-CoA reductase to react unspecifically with 2-aminobenzoyl-CoA under in vitro conditions (Boll and Fuchs 1995). We do not yet know, to what extent 2-aminobenzoyl-CoA is degraded in vivo via reductive deamination to benzoyl-CoA or via direct reduction of the aromatic ring.

Anaerobic growth of *T. aromatica* on phenylalanine, phenylacetate and phenylglyoxylate as sole substrates proceeds via a common pathway [see Schneider et al. (1997)]. Phenylalanine is first oxidised to phenylacetate by a 2-oxoglutarate-dependent transaminase, phenylpyruvate decarboxylase, and phenylacetaldehyde dehydrogenase. These enzymes were highly induced in phenylalanine-grown cells as compared to cells grown on other substrates, which contained more than tenfold lower activities (Table 2). The degradation of phenylacetate to phenylglyoxylate by *T. aromatica* involves a specific CoA ligase (Mohamed and Fuchs 1993) and a membrane-bound enzyme system catalysing the anaerobic α-oxidation of phenylacetyl-CoA to phenylglyoxylate (Mohamed et al. 1993; Schneider et al. 1997). High levels of these enzymes were present in phenylalanine- and phenylacetate-grown cells, but not in phenylglyoxylate-grown cells or in cells grown on other substrates (Tables 1, 2). Finally, phenylglyoxylate:acceptor oxidoreductase, which oxidatively decarboxylates phenylglyoxylate to benzoyl-CoA (Hirsch et al. 1998), was induced in cells grown on phenyl-

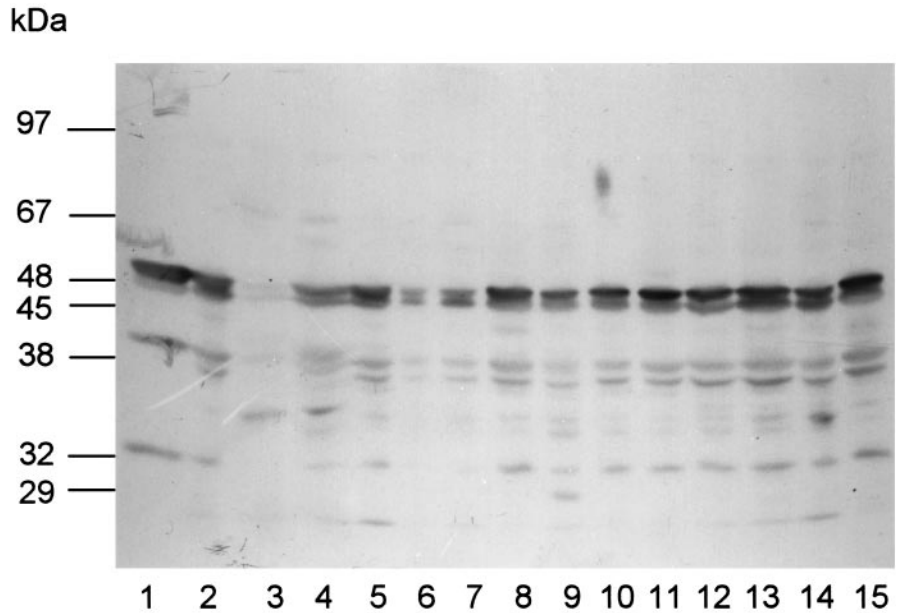

Fig. 4 Immunoblot analysis of cell free extracts of *Thauera aromatica* grown on different substrates with antiserum against purified benzoyl-CoA reductase. Polypeptides were separated in a gel containing 10% (w/v) polyacrylamide and were blotted on a nitrocellulose membrane. *Lanes 1* Purified benzoyl-CoA reductase, *2* cells grown anaerobically on benzoate, *3* cells grown aerobically on benzoate, *4–15* cells grown anaerobically on glutarate *(4)*, glutarate plus benzoate *(5)*, acetate *(6)*, acetate plus benzoate *(7)*, 2-aminobenzoate *(8)*, 3-hydroxybenzoate *(9)*, 4-hydroxybenzoate *(10)*, phenol *(11)*, phenylalanine *(12)*, phenylacetate *(13)*, phenylglyoxylate *(14)* and toluene *(15)*. The masses of the four subunits of benzoyl-CoA reductase and some marker proteins are given along the *left margin*

alanine, phenylacetate and phenylglyoxylate, but not on other substrates (Table 2).

Toluene degradation by *T. aromatica* is initiated by the addition of the methyl group of toluene to the double bond of fumarate to yield the first intermediate, benzylsuccinate (Biegert et al. 1996). This is accomplished by benzylsuccinate synthase, which has been purified from toluene-grown *T. aromatica* cells (Leuthner et al. 1998). We detected enzyme activity exclusively in toluene-grown cells (Table 2).

Enzymes involved in the central pathway of anaerobic degradation of aromatic compounds

Benzoyl-CoA reductase catalyses the reductive dearomatisation of benzoyl-CoA to cyclohexa-1,5-diene-1-carboxyl-CoA. Enzyme activity was fairly constant in cells grown on most aromatic substrates tested except for 2-aminobenzoate-grown cells, which yielded significantly increased levels (Table 2), and 3-hydroxybenzoate-grown cells, which contained only background levels of activity. Benzoyl-CoA reductase activity was absent in cells grown on the nonaromatic substrates, acetate and glutarate, and in cells grown aerobically on benzoate (Table 2).

Glutaryl-CoA dehydrogenase/decarboxylase catalyses the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA, a reaction in the β-oxidation pathway of 3-hydroxypimelyl-CoA and other dicarboxylic acids (Härtel et al. 1993). Similar levels of enzyme activity were recorded in most extracts. More than twofold higher activity was detected in glutarate-grown cells; significantly lower activities were observed in phenol- and acetate-grown cells and in cells grown aerobically on benzoate (Table 1). The citric acid cycle enzyme succinate-CoA ligase (ADP-forming) was included as a control because it is required in the final degradation of acetyl-CoA units, a common pathway in the degradation of all of the aromatic compounds and organic acids tested. Its activity was found in all extracts and varied only moderately (Table 1).

Nonaromatic CoA ligases and related enzymes

Acetate-CoA ligase activity was recorded in all extracts. The highest activity was found in cells grown anaerobically on acetate (Table 1). The lowest and highest acetate-CoA ligase activities measured (in 3-hydroxybenzoate- and acetate-grown cells, respectively) varied by a factor of 24. *T. aromatica* grew well anaerobically on glutarate as the sole carbon source, but no glutarate-CoA ligase was detected (data not shown). However, we detected a succinyl-CoA:glutarate-CoA transferase activity that was present in almost all extracts analysed and that had a five-fold-induced level in glutarate-grown cells (Table 1).

Immunological detection of catabolic enzymes in cells grown on different substrates

Benzoyl-CoA reductase.

Antiserum against purified native benzoyl-CoA reductase from *T. aromatica* was raised in rabbits and was used to

probe differently grown cells for the presence of the subunits of this enzyme. The antiserum reacted specifically with the subunits of benzoyl-CoA reductase and showed the strongest interaction with the α-subunit (Fig. 4, lane 1). The subunits were detected in cells grown anaerobically on benzoate and on all other aromatic substrates (Fig. 4, lane 2; lanes 8–15). The amounts of the γ- and δ-subunits were decreased in 3-hydroxybenzoate-grown cells, and a novel, small immunoreactive band that may represent a proteolytic degradation product (Fig. 4, lane 9) appeared. Cells grown anaerobically on nonaromatic substrates such as acetate (Fig. 4, lane 4) and glutarate (Fig. 4, lane 6) also contained subunits of benzoyl-CoA reductase, although at reduced levels. In both cell batches, the enzyme appeared to lack the δ-subunit. Cells grown on mixtures of the non-aromatic acids plus benzoate contained higher amounts of benzoyl-CoA reductase subunits as compared to cells grown on the nonaromatic acids alone (Fig. 4, lanes 5, 7). Finally, the subunits of benzoyl-CoA reductase were almost completely absent in aerobically grown cells (Fig. 4, lane 3).

4-Hydroxybenzoyl-CoA reductase

Antiserum raised in mice against the large subunit of 4-hydroxybenzoyl-CoA reductase of *T. aromatica* was used to detect its presence in cells grown on different substrates. Approximately equal amounts of the protein were detected in cells grown on phenol, 4-hydroxybenzoate and 3-hydroxybenzoate, although enzyme activity was found only in phenol- and 4-hydroxybenzoate- and not in 3-hydroxybenzoate-grown cells. No immunoreactive protein was detected in cells grown on benzoate and 2-aminobenzoate (data not shown).

CoA ligases

Antiserum raised in mice against purified anaerobic benzoate-CoA ligase from *A. evansii* (Altenschmidt et al. 1991) was used to detect benzoate-CoA ligase of *T. aromatica*. It exhibited high cross-reactivity against the anaerobic isoenzyme in *T. aromatica* cells grown under different conditions. Approximately equal band intensity was obtained in cells grown on benzoate, phenol, *p*-cresol or phenylacetate, slightly lower intensity in cells grwon on 4-hydroxybenzoate, and only a weak band in cells grown on 2-aminobenzoate. Cells grown on glutarate or aerobically on benzoate showed no immunologic reaction (data not shown). Finally, the presence of 4-hydroxybenzoate-CoA ligase was assayed with antiserum against the purified enzyme of *T. aromatica*. The enzyme was present in extracts of phenol-, *p*-cresol- and 4-hydroxybenzoate-grown cells, but not in cells grown on 2-aminobenzoate or phenylacetate (data not shown). The antisera against the two CoA ligases yielded single bands and showed very weak cross-reactivity with other polypeptides.

Discussion

Aerobic/anaerobic regulation

Several of the enzymes involved in anaerobic degradation of aromatic compounds are readily inactivated upon contact with oxygen. No activity of any of these enzymes was detected in aerobically grown cells. Strong down-regulation of synthesis of benzoyl-CoA reductase was found in response to oxygen, but trace amounts of inactive enzyme were still detected in aerobically grown cells. This may serve to maintain a backup level of enzyme for faster response to environmental shifts. Enzymes of general metabolic pathways, e. g. glutaryl-CoA dehydrogenase and succinate-CoA ligase (which are also involved in further degradation of the dearomatised and ring-opened products), glutarate-CoA transferase, and acetate-CoA ligase, are not regulated by oxygen availability. Some CoA ligase activities, namely for benzoate, 2-aminobenzoate and 3-hydroxybenzoate, were detected in aerobically grown cells. The benzoate-CoA ligases present in aerobic and anaerobic cells represent different isoenzymes that can be discriminated immunologically. The activities measured with 2-aminobenzoate and 3-hydroxybenzoate in aerobic cells may be due to unspecificity of the aerobic benzoate-CoA ligase or due to extra isoenzymes for the respective substrates. Formation of CoA-thioesters is the initial reaction in aerobic degradation of benzoate (Altenschmidt et al. 1993; Niemetz et al. 1995), 2-aminobenzoate (Buder and Fuchs 1989; Ziegler et al. 1989) and phenylacetate (M. Mohamed and G. Fuchs, unpublished work) in *A. evansii*. Detection of aerobic CoA ligase isoenzymes in *T. aromatica* indicates that the two related species use similar pathways for aerobic aromatic metabolism.

Regulation of central enzymes
of aromatic compound degradation

The subunits of benzoyl-CoA reductase were detected in all anaerobically grown cells, but synthesis was decreased in glutarate- and especially in acetate-grown cells. No enzyme activity was detectable in these cell batches, and closer inspection revealed that only the α-, β- and γ-subunits of benzoyl-CoA reductase were present; the δ-subunit was virtually lacking. Significantly lowered activity was also recorded in cells grown on one aromatic substrate, namely 3-hydroxybenzoate. These cells also exhibited an apparent decrease or degradation of the γ- and δ-subunits of benzoyl-CoA reductase. Therefore, some kind of post-translational control may be involved in regulating benzoyl-CoA reductase activity, and the δ-subunit may be a preferred target for this event. This may involve protein degradation, cofactor incorporation, or other activation or deactivation reactions. An extra band appearing in immunoblots of 3-hydroxybenzoate-grown cells (Fig. 4) might indicate protein instability.

Possible catabolite regulation effects of nonaromatic substrates on the synthesis of benzoyl-CoA reductase were analysed in cells grown on mixtures of acetate plus benzoate and glutarate plus benzoate. The nonaromatic substrates did not cause pronounced catabolite repression, whereas the presence of benzoate led to an increased synthesis of benzoyl-CoA reductase. Thus, a basal level of benzoyl-CoA reductase synthesis during anaerobic growth conditions is apparently further induced in the presence of benzoate or other aromatics. The common intermediate, benzoyl-CoA, would be a possible candidate as mediator for this induction.

Regulation of peripheral pathways

Enzymes of benzoate degradation.

Benzoate-CoA ligase was detected in all anaerobically grown cells of *T. aromatica*. Comparison of the specific activities in cells grown on different aromatic and nonaromatic substrates suggested that anaerobic benzoate-CoA ligase is a semiconstitutive enzyme during anaerobic growth. The value measured in benzoate-grown cells was not higher than in cells grown on some other substrates that are not degraded via free benzoate. Specific activities recorded in cells grown on nonaromatic substrates were among the lowest recorded. However, these effects must be interpreted with caution; they may also be caused by overlaying regulatory effects such as growth rate regulation, post-translational regulation events, or the presence of inhibitors in cell extracts (Altenschmidt et al. 1991; Altenschmidt and Fuchs 1992).

Enzymes of phenol degradation

Phenyl phosphate carboxylase activity was exclusively recorded in phenol-grown cells. These cells also contained a couple of highly induced polypeptides that were not synthesised on any other substrate. The number of induced proteins (> 5) and their degree of induction were among the most pronounced of all tested substrates. The induced polypeptides probably represent enzymes specifically involved in phenol phosphorylation and phenylphosphate carboxylation, as indicated by preliminary sequencing results of the corresponding genes (S. Breinig and G. Fuchs, unpublished work). As recorded earlier, the phenol-carboxylating system is not induced by other phenolic compounds such as *p*-cresol and 4-hydroxyphenylacetate (Dangel et al. 1991). Thus, the inductor appears to be phenol itself or a direct derivative thereof (e.g. phenyl phosphate). Phenol-grown cells also contained the enzymes required for subsequent conversion of 4-hydroxybenzoate to benzoyl-CoA. The enzyme activity pattern in phenol-, 4-hydroxybenzoate- and benzoate-grown cells is consistent with sequential induction of the enzymes required for the respective substrate (see below).

Enzymes of hydroxybenzoate degradation

T. aromatica grew on 3- and 4-hydroxybenzoate, but not on 2-hydroxybenzoate. The monohydroxybenzoate isomers are first converted to the CoA thioesters by specific CoA ligases; subsequent reductive dehydroxylation is only established for 4-hydroxybenzoyl-CoA. Substrate induction of this pathway is indicated by the presence of 4-hydroxybenzoate-CoA ligase and 4-hydroxybenzoyl-CoA reductase activity in cells grown on phenol and 4-hydroxybenzoate, but not on benzoate. Earlier data suggested sequential induction also for 4-hydroxybenzoate-CoA ligase and 4-hydroxybenzoyl-CoA reductase, 4-hydroxybenzoate being a potential inductor for synthesis of the ligase, and the CoA-thioester one for the reductase (Dangel et al. 1991). Different regulation of 4-hydroxybenzoate CoA ligase and 4-hydroxybenzoyl-CoA reductase fits well with our observation that the genes for these enzymes are not closely clustered in the genome of *T. aromatica* (Breese and Fuchs 1998).

Growth of *T. aromatica* on 3-hydroxybenzoate induced CoA ligase activities for both 3- and 4-hydroxybenzoate. The CoA ligase activity for 3-hydroxybenzoate represents a new isoenzyme, whereas the activity towards 4-hydroxybenzoate in these cells may be due either to unspecificity of 3-hydroxybenzoate-CoA ligase or to cross-induction of the 4-hydroxybenzoate-activating enzyme by 3-hydroxybenzoate. No activity of a dehydroxylating reductase for 3- or 4-hydroxybenzoyl-CoA was detected in 3-hydroxybenzoate-grown cells. This observation and the absence of benzoyl-CoA reductase activity in 3-hydroxybenzoate-grown cells raise the question whether 3-hydroxybenzoate is indeed degraded via benzoyl-CoA reductase.

Enzymes of 2-aminobenzoate degradation

2-Aminobenzoate is only a poor substrate for *T. aromatica* as compared to the related species *A. evansii*, which has originally been isolated on this compound (Braun and Gibson 1984). Its degradation is initiated by CoA-thioester formation. *T. aromatica* utilises benzoate-CoA ligase for this step. The 2-aminobenzoyl-CoA formed is then channeled into one of two parallel pathways: ring reduction or reductive deamination (U. Feil and G. Fuchs, unpublished work). Details of these pathways remain to be studied.

Enzymes of phenylalanine and phenylacetate degradation

Degradation of phenylalanine via phenylacetate and phenylglyoxylate to benzoyl-CoA appears to follow a sequential induction pattern. The enzyme activities required for degradation of phenylalanine to phenylacetate were highly induced only in cells grown on phenylalanine; those converting phenylacetate to phenylglyoxylate were detected in phenylalanine- and phenylacetate-grown cells; and only the last enzyme, phenylglyoxylate oxidoreduc-

tase, was synthesised in phenylalanine-, phenylacetate- and phenylglyoxylate-grown cells. Cells grown on other-substrates did not contain high activities of any of these enzymes.

Enzymes of toluene degradation

Benzylsuccinate synthase, the enzyme catalysing the initial step of anaerobic toluene degradation, was present only in toluene-grown cells. Synthesis of this enzyme is regulated on the transcriptional level (Leuthner et al. 1998). Several additional toluene-induced polypeptides are probably involved in the oxidation of benzylsuccinate to benzoyl-CoA. Toluene induction is apparently mediated by a two-component regulatory system whose genes are located adjacent to the structural genes of benzylsuccinate synthase (B. Leuthner and J. Heider, unpublished work).

Glutarate and acetate degradation

The two initial enzymes in glutarate metabolism, succinyl-CoA:glutarate-CoA transferase and glutaryl-CoA dehydrogenase, were found in all cell batches and were approximately three- to fivefold induced in glutarate-grown cells. Likewise, AMP-forming acetate-CoA ligase was constitutively synthesised but was most active in acetate-grown cells. Induction of these enzymes by acetate and glutarate probably proceeds similarly to the mechanism outlined above for benzoate: a basal level of expression of responsive genes appears to be boosted by high levels of the free acids or, more likely, of the CoA thioesters.

Acknowledgements This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. Thanks are also due to T. Biegert and R. Brackmann for their contributions in the initial stages of this work.

References

Altenschmidt U, Fuchs G (1992) Novel aerobic 2-aminobenzoate metabolism. Purification and characterisation of 2-aminobenzoyl-coenzyme A synthetase, localisation of the gene on a plasmid, and cloning and sequencing of the gene. Eur J Biochem 205:721–727

Altenschmidt U, Oswald B, Fuchs G (1991) Purification and characterisation of benzoate-coenzyme A ligase and 2-aminobenzoate-coenzyme A ligases from denitrifying *Pseudomonas* sp. J Bacteriol 173:5494–5501

Altenschmidt U, Oswald B, Steiner E, Herrmann H, Fuchs G (1993) New aerobic benzoate oxidation via benzoyl-CoA and 3-hydroxybenzoyl-CoA in a denitrifying *Pseudomonas* sp. J Bacteriol 175:4851–4858

Anders A, Kaetzke A, Kämpfer P, Ludwig W, Fuchs G (1995) Taxonomic position of aromatic degrading denitrifying pseudomonad strains K172 and KB740 and their description as new members of the genera *Thauera*, *T. aromatica* sp. nov., and *Azoarcus*, *A. evansii* sp. nov., respectively, members of the beta subclass of Proteobacteria. Int J Syst Bacteriol 45:327–333

Biegert T, Altenschmidt U, Eckerskorn C, Fuchs G (1993) Enzymes of anaerobic metabolism of phenolic compounds. 4-Hydroxybenzoate-CoA ligase from a denitrifying *Pseudomonas* species. Eur J Biochem 213:555–561

Biegert T, Fuchs G, Heider J (1996) Evidence that 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

Brackmann R, Fuchs G (1993) Enzymes of anaerobic metabolism of phenolic compounds. 4-Hydroxybenzoate-CoA reductase (dehydroxylating) from a denitrifying *Pseudomonas* species. Eur J Biochem 213:563–571

Braun K, Gibson DT (1984) Anaerobic degradation of 2-aminobenzoate (anthranilic acid) by denitrifying bacteria. Appl Env Microbiol 48:102–107

Breese K, Fuchs G (1998) 4-Hydroxybenzoyl-CoA reductase (dehydroxylating) from the denitrifying bacterium *Thauera aromatica*. Prosthetic groups, electron donor and genes of a new member of molybdenum-flavin-iron-sulfur-proteins. Eur J Biochem 251:916–923

Buder R, Fuchs G (1989) 2-Aminobenzoyl-CoA monoxygenase/reductase, a novel type of flavoenzyme: purification and some properties of the enzyme. Eur J Biochem 185:629–635

Dangel W et al. (1991) Differential expression of enzyme activities initiating anoxic metabolism of various aromatic compounds via benzoyl-CoA. Arch Microbiol 155:256–262

Gross GG, Zenk MH (1966) Darstellung und Eigenschaften von Coenzym A-Thioestern substituierter Zimtsäuren. Z Naturforsch 21b:683–690

Härtel U, Ecker E, Koch J, Fuchs G, Linder D, Buckel W (1993) Purification of glutaryl-CoA dehydrogenase from *Pseudomonas* sp., an enzyme involved in the anaerobic degradation of benzoate. Arch Microbiol 159:174–181

Harwood CS, Parales RE (1996) The β-ketoadipate pathway and the biology of self-identity. Annu Rev Microbiol 50:553–590

Heider J, Fuchs G (1997) Anaerobic metabolism of aromatic compounds. Eur J Biochem 243:577–596

Hirsch W, Schägger H, Fuchs G (1998) Phenylglyoxylate:NAD^+ oxidoreductase (CoA benzoylating), a new enzyme of anaerobic phenylalanine metabolism. Eur J Biochem 251:907–915

Koch J, Fuchs G (1992) Enzymatic reduction of benzoyl-CoA to alicyclic compounds, a key reaction in anaerobic aromatic metabolism. Eur J Biochem 205:195–202

Lack A, Fuchs G (1992) Carboxylation of phenylphosphate by phenol carboxylase, an enzyme system of anaerobic phenol metabolism. J Bacteriol 174:3629–3636

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

Lehman TC, Hale DE, Bhala A, Thorpe C (1990) An acyl-coenzyme A dehydrogenase assay using ferricenium ion. Anal Biochem 186:280–284

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

Lochmeyer C, Koch J, Fuchs G (1992) Anaerobic degradation of 2-aminobenzoic acid (anthranilic acid) via benzoyl-CoA and cyclohex-1-enecarboxyl-CoA in a denitrifying bacterium. J Bacteriol 174:3621–3628

Mohamed ME, Fuchs G (1993) Purification and characterisation of phenylacetate-coenzyme A ligase from a denitrifying *Pseudomonas* sp., an enzyme involved in the anaerobic degradation of phenylacetate. Arch Microbiol 159:554–562

Mohamed ME, Seyfried B, Tschech A, Fuchs G (1993) Anaerobic oxidation of phenylacetate and 4-hydroxyphenylacetate to benzoyl-CoA and CO_2 in denitrifying *Pseudomonas* sp. Evidence for an α-oxidation mechanism. Arch Microbiol 159 : 563–573

Niemetz R, Altenschmidt U, Bruckner S, Fuchs G (1995) Benzoyl-coenzyme A 3-monoxygenase, a flavin-dependent hydroxylase: purification, some properties and its role in aerobic benzoate oxidation via gentisate in a denitrifying bacterium. Eur J Biochem 227 : 161–168

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250 : 4007–4021

Rao P, Trancis N, Metcalfe D (1983) An avidin-biotin micro ELISA for rapid measurement of total allergen-specific human IgE. J Immunol Methods 57 : 71–85

Schachter D, Taggart JV (1976) Benzoyl coenzyme A and hippurate synthesis. J Biol Chem 203 : 925–933

Schneider S, Mohamed ME, Fuchs G (1997) Anaerobic metabolism of L-phenylalanine via benzoyl-CoA in the denitrifying bacterium *Thauera aromatica*. Arch Microbiol 168 : 310–320

Tschech A, Fuchs G (1987) Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. Arch Microbiol 148 : 213–217

Ziegler K, Buder R, Winter J, Fuchs G (1989) Activation of aromatic acids and aerobic 2-aminobenzoate metabolism in a denitrifying *Pseudomonas* strain. Arch Microbiol 151 : 171–176