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ORIGINAL PAPER

Enzymes of anaerobic ethylbenzene and *p*-ethylphenol catabolism in '*Aromatoleum aromaticum*': differentiation and differential induction

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Abstract The denitrifying bacterium '*Aromatoleum aromaticum*' strain EbN1 is one of the best characterized bacteria regarding anaerobic ethylbenzene degradation. EbN1 also degrades various other aromatic and phenolic compounds in the absence of oxygen, one of them being *p*-ethylphenol. Despite having similar chemical structures, ethylbenzene and *p*-ethylphenol have been proposed to be metabolized by completely separate pathways. In this study, we established and applied biochemical and molecular biological methods to show the (almost) exclusive presence and specificity of enzymes involved in the respective degradation pathways by recording enzyme activities, complemented by heme staining, immuno- and biotin-blotting analyses. These combined results substantiated the predicted *p*-ethylphenol degradation pathway. The identified enzymes include a heme *c*-containing *p*-ethylphenol-hydroxylase, both an (*R*)- and an (*S*)-specific alcohol dehydrogenase as well as a novel biotin-dependent carboxylase. We also establish an activity assay for benzoylacetate-CoA ligases likely being involved in both metabolic pathways.

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Introduction

The denitrifying β-proteobacterium '*Aromatoleum aromaticum*' strain EbN1 was originally isolated as an anaerobic ethylbenzene-degrading bacterium (Rabus and Widdel 1995). Together with the closely related strains PbN1, EB1 and EbS7, it represents one of the first and still very rare pure cultures exhibiting this phenotype (Rabus and Widdel 1995; Ball et al. 1996; Heider et al. 1999; Kniemeyer et al. 2003). Since strain EbN1 was found to utilize an extraordinarily broad range of diverse aromatic compounds as substrates, it was selected as model organism and the first anaerobic aromatics-degrading bacterium to have its genome sequenced (Rabus and Widdel 1995; Rabus et al. 2005). The annotation included genes coding for the enzymes of ten anaerobic and four aerobic pathways of aromatic compound degradation that had previously been identified in other bacteria, and still left potential for further unknown metabolic pathways (Heider and Fuchs 1997a, b; Boll et al. 2002; Rabus et al. 2005).

The initial step of anaerobic ethylbenzene degradation is an oxygen-independent hydroxylation to (*S*)-1-phenylethanol catalyzed by the oxygen-labile molybdoenzyme ethylbenzene dehydrogenase (EbDH) (Fig. 1a; Ball et al. 1996; Rabus and Heider 1998; Kniemeyer and Heider 2001a). The subsequent step is an NAD-dependent oxidation to acetophenone catalyzed by (*S*)-1-phenylethanol dehydrogenase (PEDH), a member of the short-chain dehydrogenase/reductase (SDR) enzyme family (Ball et al. 1996; Rabus and Heider 1998; Johnson et al. 2001; Kniemeyer and Heider 2001b). Further degradation involves an

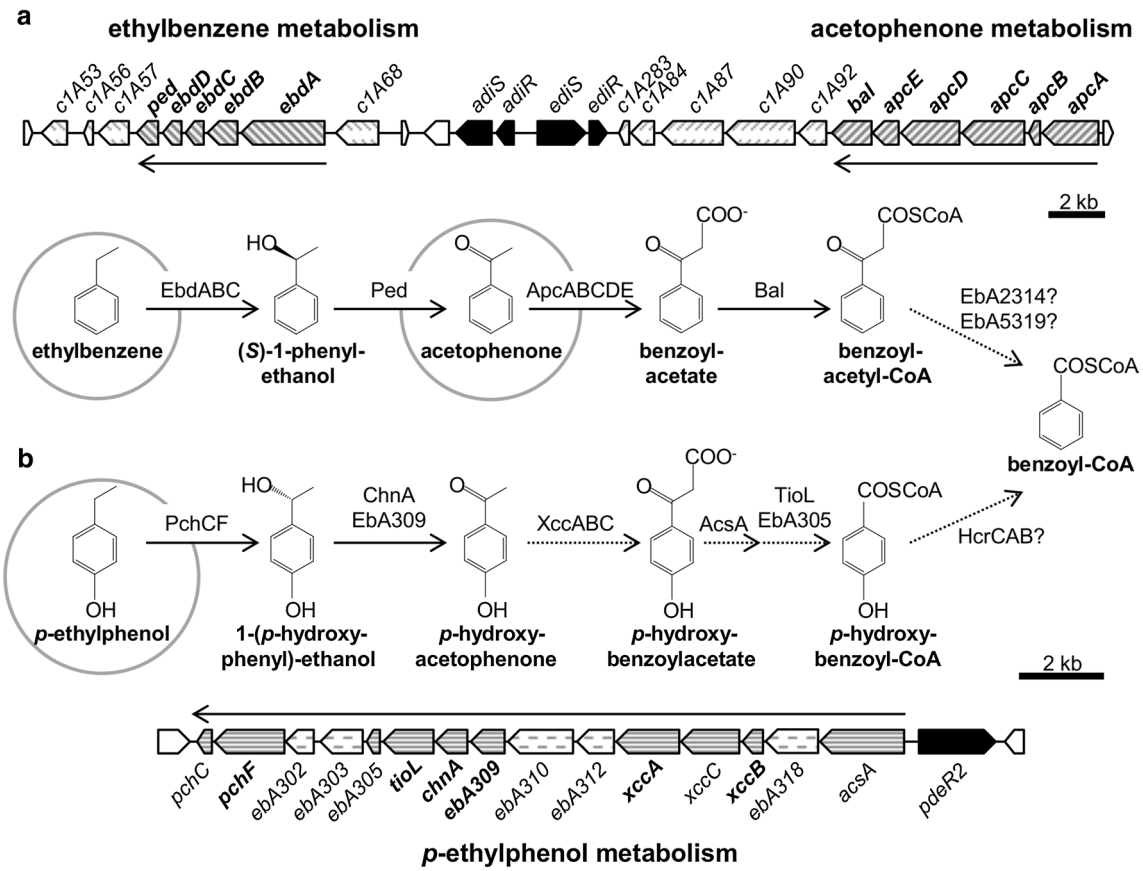

Fig. 1 Overview of the genes and gene products involved in anaerobic degradation of ethylbenzene (**a**) and *p*-ethylphenol (**b**) in '*Aromatoleum aromaticum*' (modified from Rabus et al. 2002, 2014; Wöhlbrand et al. 2008). *Broken arrows* indicate hypothetical reactions which are not yet biochemically confirmed. Different functions of the gene products are indicated by different hatching patterns: *black* regulation, *diagonal stripes* involved in ethylbenzene or acetophenone degradation, *horizontal stripes* involved in *p*-ethylphenol degradation and *broken lines* proteins of unknown function. Substrate-induced proteins previously identified by proteomic analysis are indicated in *bold type*. **a** Ethylbenzene catabolic gene cluster (accession numbers of gene products: CAI07425 for C1A53 to CAI07450 for ApcA). *Names* Ped: (*S*)-1-phenylethanol dehydrogenase, EbdABCD: ethylbenzene dehydrogenase, AdiSR (Tcs1/Tcr1): predicted regulation system for acetophenone degradation, EdiSR (Tcs2/Tcr2): predicted regulation system for ethylbenzene degradation, Bal: benzoylacetate-CoA ligase, ApcABCDE: acetophenone carboxylase, and EbA2314 or EbA5319: potential β-ketothiolases (accession numbers CAI07405, CAI09148, respectively; not localized in the gene cluster shown). **b** *p*-Ethylphenol catabolic gene cluster (accession numbers of gene products: CAI06276 for PchC to CAI06290 for AcsA; CAI06292 for PdeR2). Names: PchCF: *p*-ethylphenol methylenehydroxylase, TioL and EbA305: subunits of a predicted thiolase, ChnA and EbA309: alcohol dehydrogenases (SDR family), XccABC: predicted subunits of a biotin-dependent carboxylase, AcsA: CoA ligase, PdeR2: predicted XylR-type regulator, and HcrABC: *p*-hydroxybenzoyl-CoA reductase (accession numbers CAI008160 to CAI08162; not localized in the gene cluster shown)

ATP-dependent carboxylation at the methyl group to benzoylacetate by acetophenone carboxylase (Apc), activation to benzoylacetyl-coenzyme A (CoA) by a predicted benzoylacetate-CoA ligase (Bal) and thiolytic cleavage to benzoyl-CoA and acetyl-CoA by a so far unknown thiolase (Ball et al. 1996; Rabus and Heider 1998; Champion et al. 1999; Rabus et al. 2002; Jobst et al. 2010). The final degradation of benzoyl-CoA follows the widespread anaerobic benzoyl-CoA pathway (Harwood et al. 1998; Carmona et al. 2009; Fuchs et al. 2011). The genes coding for the ethylbenzene catabolic enzymes are clustered together and appear to be organized in two operons containing either the genes for the subunits of EbDH (*ebdABCD*) and PEDH (*ped*) or those of the Apc subunits (*apcABCDE*) and Bal (*bal*), respectively (Fig. 1a). The gene cluster also contains genes coding for two two-component systems that are predicted to be involved in regulating the synthesis of the enzymes. Based on the results of growth studies, proteomic experiments and bioinformatics analysis, the two catabolic operons have been predicted to be sequentially induced by exposure of the cells to ethylbenzene and acetophenone (as external substrate or transient metabolic intermediate). Both two-component systems have been implicated in mediating this regulation in response to either ethylbenzene (*ediSR* genes) or acetophenone (*adiRS* genes) (Fig. 1a) (Rabus et al. 2002).

Further proteomic analyses on EbN1 revealed several new catabolic pathways (Hufnagel and Rabus 2006; Wöhlbrand et al. 2007, 2008; Ebenau-Jehle et al. 2012; Trautwein et al. 2012). A surprising result of these studies was the presence of an apparent separate and specific degradation pathway for *p*-ethylphenol (Fig. 1b; Wöhlbrand et al. 2008), since the enzymes initiating anaerobic ethylbenzene metabolism also catalyze the transformation of *p*-ethylphenol with very high activities (Kniemeyer and Heider 2001a, b; Knack et al. 2012). The proposed *p*-ethylphenol degradation pathway shows similarity to ethylbenzene metabolism in the first two steps, which involve an initial hydroxylation followed by alcohol dehydrogenation to *p*-hydroxyacetophenone (Wöhlbrand et al. 2008). However, hydroxylation appears to be catalyzed by a flavocytochrome *c*-type *p*-ethylphenol methylenehydroxylase (EpMH) related to *p*-cresol methylhydroxylase, rather than by a molybdenum enzyme. A corresponding EpMH involved in aerobic *p*-ethylphenol degradation in *Pseudomonas putida* strain JD1 has been purified and characterized previously (McIntire et al. 1984, 1985; Reeve et al. 1989). While the sequence of this enzyme is not yet available, the *pchCF* genes in the proposed *p*-ethylphenol catabolic operon are very likely to code for an orthologous EpMH (Fig. 1; Rabus et al. 2005; Wöhlbrand et al. 2008; Rabus et al. 2014). The derived gene products show 72 % sequence identity to the subunits of a putative EpMH orthologue in *Pseudomonas alkylphenolia* and >50 % identity to orthologues in many other aerobic bacteria, but only <38 % identity to the corresponding subunits of *p*-cresol methylhydroxylases. The further degradation of *p*-hydroxyacetophenone is still unclear, but it has been proposed that a predicted biotin-dependent carboxylase encoded in the *eph* operon may be involved in carboxylating the ketone, which would mark a novel reaction type among the biotin-dependent carboxylases (Wöhlbrand et al. 2008). After activation of the product by a CoA ligase and thiolytic cleavage, *p*-hydroxybenzoyl-CoA would be produced as intermediate, which would then be reduced to benzoyl-CoA by a *p*-hydroxybenzoyl-CoA reductase (Wöhlbrand et al. 2008). The predicted *eph* operon seems to be regulated in response to substrate availability by the product of the adjacent *pdeR2* (*ebA324*) gene which codes for a putative σ^{54}-dependent XylR-type transcriptional regulator (Rabus et al. 2005; Wöhlbrand et al. 2008).

This work extends previous studies based on proteomic analyses in '*A. aromaticum*' (for overview see Rabus et al. 2014) with biochemical and enzymatic data. We established biochemical and molecular biological methods to provide insights into the distinct phenotypic patterns and key enzymes of cells degrading either ethylbenzene or *p*-ethylphenol (Fig. 1), using cells grown on five different carbon and energy sources. Additionally, these data were validated by heme staining, immuno- and biotin-blotting analyses.

Materials and methods

Growth conditions and preparation of cell-free extracts

'*Aromatoleum aromaticum*' strain EbN1 was grown anaerobically at 28 °C as described elsewhere (Rabus and Widdel 1995; Rabus and Heider 1998). Benzoate, ethylbenzene, acetophenone, *p*-ethylphenol or phenol were used as carbon and energy sources (at 1–5 mM). Every culture was adapted to anaerobic growth with the respective substrate for at least four passages. For cultures growing on ethylbenzene, acetophenone and *p*-ethylphenol, 2 % (v/v) paraffin oil (low viscosity; Roth, Karlsruhe, Germany) was added to the medium as inert carrier phase to protect the cultures against the potentially toxic substrates. Nitrate was added to the medium as terminal electron acceptor at an initial concentration of 5 mM. '*A. aromaticum*' was routinely cultivated in 2-l bottles or in a 100-l fermenter under N_2-atmosphere. Bacterial growth was followed by measuring optical density at 578 nm and the consumption of nitrate. Cultures grown in 100-l scale were run in fed-batch mode with a growth-limiting and exponentially increasing feeding rate of nitrate and a discontinuous supply of electron donor. Cells were routinely harvested by centrifugation during the exponential growth phase at an optical density (OD_{578}) around 4.0. Cell yields under these conditions range between 0.8 and 1.2 g l^{-1} (wet mass) per $OD_{578} = 1$ (see Rabus and Heider 1998). The cells were immediately frozen in liquid nitrogen and stored at −80 °C.

Cells of *Escherichia coli* strain DH5α were grown aerobically at 37 °C in LB medium (Sambrook and Russell 2001). If required, the growth medium was solidified with 1.5 % (w/v) agar. For selection of maintenance of cells carrying recombinant plasmids, ampicillin was added to the media at a final concentration of 100 µg ml^{-1}.

For extract preparation, frozen cells were resuspended in an equal volume of 10 mM Tris/HCl buffer pH 7.5 containing 0.05 mg lysozyme and 0.05 mg DNase I per ml. The cell suspensions were passed three times through a French pressure cell press at 110 MPa. Cell debris and membranes were removed by ultracentrifugation at 125,000×*g* and 4 °C for 1 h. The cell-free extracts (soluble fractions) were used immediately or stored in aliquots with added 10 % (v/v) glycerol at –20 °C.

Production of recombinant proteins

The genes *chnA* (*ebA307*) and *ebA309* of '*A. aromaticum*' were amplified via PCR using the oligonucleotide primers: chnA_for (TGCTGCTCGAAGGGAAAACCGC) and chnA_rev (GAGGATCATCGTGCGAGGTAGC), respectively, ebA309_for (TGAAGCAGAATTTGTCTGG) and ebA309_rev (GGTCAGCTGATCGTCATTCC). The

amplicons were cloned blunt-end into the *Box*I restriction site of expression plasmid pASK-IBA5plus (IBA GmbH, Goettingen, Germany) and selected for correct orientation and fusion of a plasmid-bourne *Strep*-tag at the N-terminus of the gene products. The respective genes were overexpressed in *E. coli* DH5α. After harvesting and lysing the recombinant cells, the overproduced proteins were purified from the centrifuged extract via *Strep*-tag affinity chromatography as recommended by the supplier (IBA GmbH, Goettingen, Germany).

Electrophoretic methods and immunoblotting

Cell-free extracts of '*A. aromaticum*' (50 μg of protein) were separated by discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) using 11.5 % and 16.5 % (w/v) polyacrylamide gels as described elsewhere (Laemmli 1970). Resolved proteins were either stained with Coomassie Brilliant Blue R-250 or immediately blotted onto nitrocellulose membranes (Whatman, Dassel, Germany) using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell as recommended by the supplier (Bio-Rad, Munich, Germany). Immunoblot analysis were performed using antisera raised in rabbits against the purified core enzyme of Apc (1:5000, BioScience, Göttingen, Germany) or against purified EbDH (1:2500, BioScience, Göttingen, Germany). The purification protocols for the two enzymes were described previously (Kloer et al. 2006; Jobst et al. 2010). Horseradish peroxidase (HRP)-coupled antirabbit IgG was used as secondary antibody (1:2000, Cell Signaling Technology, Danvers, MA, USA). Biotin-containing proteins were detected by treating blots with HRP-coupled avidin (1:750, eBioscience, San Diego, CA, USA). All blots were developed using a colorimetric assay for HRP with H_2O_2 and *p*-chloro-1-naphthol (Gallagher et al. 1997).

Heme staining

Staining was performed as described previously (Thomas et al. 1976), except using 1.25 mM 3,3′,5,5′-tetramethylbenzidine. Cell-free extracts (100 μg of protein) and commercially available horse heart cytochrome *c* (0.5 μg; Roth, Karlsruhe, Germany) were separated by SDS-PAGE using a 16.5 % (w/v) polyacrylamide gel, blotted on nitrocellulose and incubated in the staining mixture in the dark at room temperature.

Enzyme assays

Ethylbenzene dehydrogenase

EbDH activity was observed photometrically at 30 °C in 100 mM Tris/HCl buffer pH 7.5 containing 200 μM ferricenium tetrafluoroborate (Kniemeyer and Heider 2001a) or 1 mM phenazine methosulfate (PMS) and 100 μM 2,6-dichlorophenolindophenol (DCPIP) as electron acceptors (McIntire et al. 1984; Reeve et al. 1989). The reduction of ferricenium was detected at 290 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) using UV cuvettes, while reduction of DCPIP was detected at 600 nm ($\varepsilon = 21\ mM^{-1}\ cm^{-1}$). The photometric assay was started by addition of different substrates (final concentration 100 and 200 μM, respectively) from stock solutions prepared in *tert*-butanol as non-reactive solvent.

p-Ethylphenol methylenehydroxylase

Similar to the photometric assay of EbDH, the activity of EpMH was measured at 25 °C in 50 mM Tris/HCl buffer pH 7.6 under anaerobic conditions as described previously (McIntire et al. 1985; Reeve et al. 1989). In addition, a modified assay was established by applying 200 μM ferricenium tetrafluoroborate instead of PMS and DCPIP as electron acceptors (Kniemeyer and Heider 2001a).

Phenylethanol dehydrogenase

Activity of (*S*)- and (*R*)-1-phenylethanol dehydrogenases were measured at 30 °C in 100 mM Tris/HCl buffer pH 8.0 containing 2 mM $MgCl_2$ and 0.5 mM NAD^+ as electron acceptor (Kniemeyer and Heider 2001b). The reaction was followed at 365 nm ($\varepsilon = 3.4\ mM^{-1}\ cm^{-1}$). The photometric assay was started by addition of 1 mM (*S*)- or (*R*)-phenylethanol from stock solutions prepared in *tert*-butanol. Samples in which the substrates were omitted, but *tert*-butanol was added, served as controls.

Enzyme activities of the purified *chnA* and *ebA309* gene products were measured in either 50 mM potassium phosphate buffer pH 7.5 (ChnA) or 50 mM Tris/HCl buffer pH 8.5 (EbA309) containing 0.5 mM NAD^+ and 1–2 μg of purified enzyme. The assays (0.75 ml) were started by addition of 1 mM (*R*)- or (*S*)-phenylethanol. The reverse reactions of both enzymes were determined in 50 mM MES buffer pH 6.0 containing 0.5 mM NADH and 1–2 μg of purified enzyme, and started by addition of 1 mM *p*-hydroxyacetophenone.

Benzoylacetate reductase

Benzoylacetate reductase activity was determined photometrically by monitoring the oxidation of NADH at 365 nm ($\varepsilon = 3.4\ mM^{-1}\ cm^{-1}$) in 100 mM Tris/HCl buffer pH 7.8 containing 5 mM $MgCl_2$, 0.4 mM NADH and cell-free extract (0.1–0.25 mg protein). The reaction was started by addition of 0.5 mM benzoylacetate. Benzoylacetate was freshly prepared from its ethyl ester (5 mg), which was

dissolved in 1 ml of 1 M NaOH, hydrolyzed by incubation at room temperature for 30 min and neutralized with HCl.

Carboxylases

Carboxylase activities were measured via substrate-dependent incorporation of [^{14}C]bicarbonate into non-volatile acid-stable products (Jobst et al. 2010). The assay was performed in 100 mM MOPS/KOH buffer pH 6.5 containing 40 mM $KHCO_3$, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM NH_4Cl, 5 mM ATP, 37 kBq of NaH[^{14}C]O_3 (0.925 Bq × nmol^{-1} specific radioactivity per assay) and cell-free extract (0.3–0.6 mg protein). The reaction was initiated by adding the different substrates (1 mM end concentrations) to the standard assay mixture. Immediately after initiation, a control sample of 300 µl was withdrawn and the carboxylase reaction was stopped by adding 90 µl of 5 M $NaHSO_4$ to reach a final pH of 2.0 and precipitate the protein. After 5 and 10 min, further samples of 300 µl were withdrawn and acidified. Nonfixed volatile $^{14}CO_2$ was removed by vigorously shaking in open scintillation vials for 3 h at room temperature. The remaining radioactivity was determined by liquid scintillation counting.

Coenzyme A ligases

All coenzyme A (CoA) ligase activities except that of benzoylacetate-CoA ligase (Bal) were determined using an indirect NADH-linked photometric assay as described previously (Ziegler et al. 1987; Schühle et al. 2003). Since we observed significant activities of NADH-dependent benzoylacetate reduction in most extracts, the activity of Bal was followed directly for formation of benzoylacetyl-CoA by recording the increase in absorption at 300 nm ($\varepsilon = 2.08$ mM^{-1} cm^{-1}), which has been proposed to arise from the formation of a Mg^{2+}-chelate complex with the enolate form of benzoylacetyl-CoA (Stern 1956). Control experiments for this assay were performed with benzoate, which showed a very similar absorption increase at 300 nm and yielded almost identical values of benzoate-CoA ligase activities in the different extracts as from the coupled assay. The assay mixture (0.75 ml) contained 100 mM Tris/HCl buffer pH 7.8, 5 mM $MgCl_2$, 1 mM ATP, 0.4 mM CoA, 2 mM DTT, 0.1–0.25 mg of cell-free extract and 1 mM phosphoenolpyruvate, myokinase and pyruvate kinase for ATP regeneration. The reaction was started by addition of 0.5 mM benzoylacetate (prepared from the ethyl ester as described above) or 0.5 mM benzoate, respectively. The commercially acquired ethyl benzoylacetate (Alfa Aesar, Karlsruhe, Germany) showed a significant contamination with benzoate (about 30 %, as detected by high-performance liquid chromatography). This resulted in a two-phase reactivity in the enzyme assays with benzoylacetate, where an initial fast reaction consumed the contaminating benzoate and Bal activity was only detected as a slower second phase 5–10 min after starting the reactions.

Other methods

Determination of protein concentrations was done by the Coomassie dye binding assay with bovine serum albumin as a standard (Bradford 1976). The identities of proteins separated by SDS-PAGE were determined by mass spectrometry using a 4800 Proteomics Analyzer (MDS Sciex, Concord, ON, Canada). MS data were evaluated against an in-house database using Mascot embedded into GPS explorer software (MDS Sciex, Concord, ON, Canada).

Results

Ethylbenzene dehydrogenase and *p*-ethylphenol methylenehydroxylase

To differentiate between EbDH (Kniemeyer and Heider 2001a) and EpMH activities (Wöhlbrand et al. 2008), we performed enzymes assays for hydroxylation of ethylbenzene, *p*-ethylphenol, indane and 5′-hydroxyindane with either ferricenium tetrafluoroborate or phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCPIP) as artificial electron acceptors (McIntire et al. 1985; Reeve et al. 1989; Kniemeyer and Heider 2001a). We detected an ethylbenzene-hydroxylating enzyme in extracts of cells grown on ethylbenzene, with specific activities of 5.0 ± 1.2 nmol min^{-1} (mg protein)$^{-1}$, whereas none of the extracts grown on other substrates showed any measurable activity with ethylbenzene, confirming this enzyme to be EbDH (Table 1). The ethylbenzene-hydroxylating extract showed even higher activities with *p*-ethylphenol and the bicyclic aromatic compound indane as alternative substrates, but no activity with 5′-hydroxyindane. Analysis of the protein patterns of the different extracts after SDS-PAGE confirmed the presence of the large α-subunit of EbDH (96 kDa) only in extracts of ethylbenzene-grown cells (Fig. 2a). The presence of EbDH was additionally confirmed by immunoblotting of extracts separated on an SDS gel, using antiserum raised against purified EbDH (Fig. 2d). The antiserum strongly reacted with the two larger subunits of EbDH (96 and 43 kDa), while the small subunit was not detected under the conditions used (Fig. 2d, lane EB). Cells grown on acetophenone, *p*-ethylphenol or phenol showed no immunological reaction, whereas benzoate-grown cells showed a cross-reacting band unrelated to the EbDH subunits.

To distinguish between the activities of EpMH and EbDH, we set up photometric assays with the previously

Table 1 Activities of ethylbenzene dehydrogenase (EbDH) and *p*-ethylphenol methylenehydroxylase (EpMH) in cell-free extracts of *A. aromaticum* grown under denitrifying conditions on different substrates

Growth substrate	Specific activity with ferricenium tetrafluoroborate				PMS–DCPIP
	Ethylbenzene	Indane	*p*-Ethylphenol	5′-Hydroxyindane	*p*-Ethylphenol
Benzoate	<0.1	<0.1	3.0 ± 0.5	<0.1	1.3 ± 0.2
Acetophenone	<0.1	<0.1	<0.1	<0.1	nd
Ethylbenzene	5.0 ± 1.2	8.2 ± 0.4	9.3 ± 0.6	<0.1	2.6 ± 0.2
p-Ethylphenol	<0.1	0.7 ± 0.1	32.2 ± 0.3	4.4 ± 0.2	5.2 ± 0.4
Phenol	<0.1	<0.1	11.5 ± 0.2	<0.1	nd

Specific activities are given in nmol per min and mg of protein. The values given are the average of at least three parallel measurements including the standard deviation

nd not determined

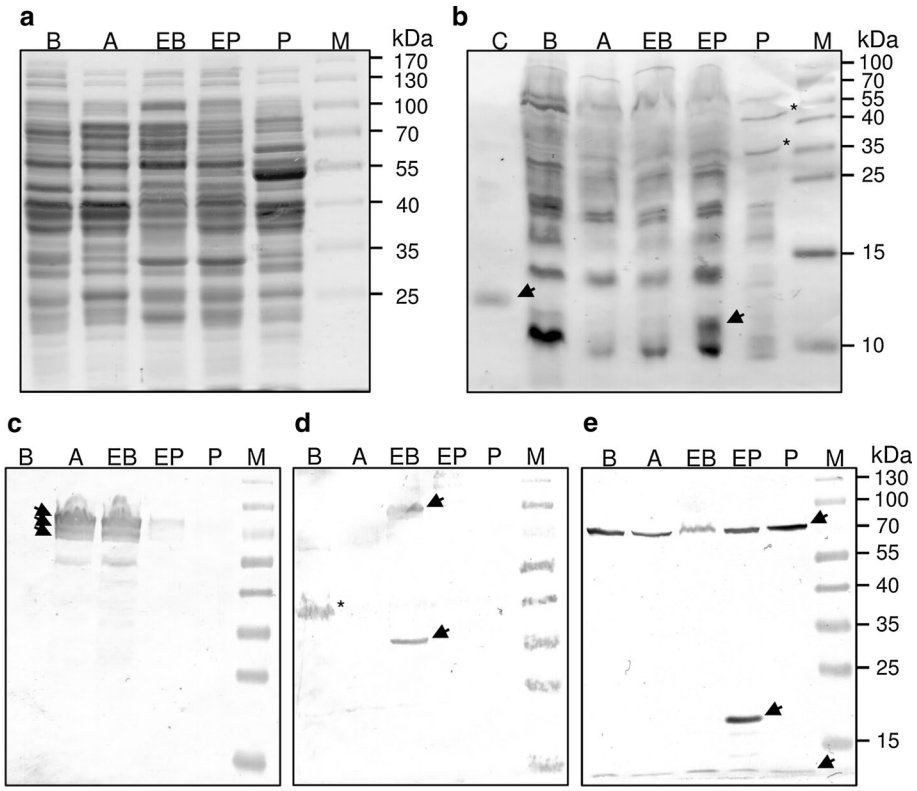

Fig. 2 Polypeptide patterns, heme staining and immunoblot analysis of cell-free extracts of '*A. aromaticum*' grown under denitrifying conditions on different substrates. *Lanes* '*A. aromaticum*' grown on benzoate (*B*), acetophenone (*A*), ethylbenzene (*EB*), *p*-ethylphenol (*EP*) and phenol (*P*). Molecular masses of the protein standard (*M*) are indicated along the *right margin*. **a** Coomassie staining. **b** Heme staining. *Arrows* identify the cytochrome *c* control (0.5 µg; *C*) and the heme *c*-containing small subunit of *p*-ethylphenol methylenehydroxylase PchC (12.4 kDa). The *asterisks* indicate two cross-reacting phenol-induced gene products of unknown function. **c** Immunoblot analysis with antiserum raised against purified acetophenone carboxylase. The recognized subunits ApcC (87 kDa), ApcD (75 kDa) and ApcA (70 kDa) are indicated by *arrows*. **d** Immunoblot analysis with antiserum raised against purified EbDH. The recognized subunits EbdA (96 kDa) and EbdB (43 kDa) are indicated by *arrows*. The *asterisk* indicates an unidentified cross-reacting polypeptide in cells grown on benzoate. **e** Biotin-blot analysis with HRP-coupled avidin. *Arrows* indicate two constitutive biotin-containing proteins identified as alpha subunit of propionyl-CoA carboxylase PccA (73.1 kDa) and biotin carrier protein of acetyl-CoA carboxylase AccB (16 kDa), as well as an additional *p*-ethylphenol-induced protein identified as biotin carrier protein XccB (17.6 kDa)

introduced substrate analogs. Additionally, we compared the results of *p*-ethylphenol-hydroxylation assays with either ferricenium or a mixture of PMS and DCPIP as electron acceptors (McIntire et al. 1985; Kniemeyer and Heider 2001a). EpMH activities of 32.2 ± 0.3 and 5.2 ± 0.4 nmol min^{-1} (mg protein)$^{-1}$ were recorded with

extracts of *p*-ethylphenol-grown cells in ferricenium- and PMS/DCPIP-coupled assays, respectively. These results indicate a similar electron acceptor preference of EpMH as found for EbDH (Table 1). However, EpMH appears to be more specific toward its natural substrate than EbDH, since extracts of *p*-ethylphenol-grown cells showed no activity with the hydrocarbon analog ethylbenzene and only a very low activity of 0.7 ± 0.1 nmol min^{-1} (mg protein)$^{-1}$ with indane. In contrast, the phenolic analog 5′-hydroxyindane was hydroxylated with a specific activity of 4.4 ± 0.1 nmol min^{-1} (mg protein)$^{-1}$ with extracts of *p*-ethylphenol-grown cells. The assays performed also yielded unexpected *p*-ethylphenol-hydroxylating background activities in benzoate- and phenol-grown cells (Table 1). The observed increased activity determined in ethylbenzene-grown cells is expected by turning over *p*-ethylphenol as alternative substrate by EbDH as mentioned above.

The predicted EpMH consists of two subunits, of which the smaller subunit PchC (12.4 kDa) contains a heme *c* cofactor (Wöhlbrand et al. 2008). Therefore, presence of this enzyme in *p*-ethylphenol-grown cells was corroborated by heme staining of '*A. aromaticum*' extracts separated by SDS-PAGE and blotted on a nitrocellulose membrane (Fig. 2b). Among many constitutively expressed heme *c*-containing proteins present in all tested extracts, we determined an additional protein of the expected size, which was only present in *p*-ethylphenol-grown cells (Fig. 2b, lane EP).

(*S*)- and (*R*)-1-phenylethanol dehydrogenases

It is known that EbDH produces the (*S*)-enantiomer of 1-phenylethanol (Johnson and Spormann 1999; Kniemeyer and Heider 2001b; Knack et al. 2012), whereas EpMH is expected to produce (*R*)-1-(*p*-hydroxyphenyl)ethanol as deduced from the orthologous EpMH from *P. putida* JD1 (Reeve et al. 1990). The alcohol dehydrogenases of the SDR family catalyze the following oxidation to ketones and usually turn over many different alternative substrates, as shown for the *ped* gene product or an SDR enzyme from a *Rhodococcus* species (Dudzik et al. 2014; Wang et al. 2014). Therefore, we investigated their activities and enantiospecificities in the various extracts by using either (*S*)- or (*R*)-1-phenylethanol as proxies for the commercially unavailable 1-(*p*-hydroxyphenyl)ethanol enantiomers. Indeed, we determined a 1.4-fold higher specific activity of (*S*)-PEDH compared with (*R*)-PEDH in cells grown on ethylbenzene, while control extracts of cells grown on benzoate showed no detectable activities of either (*S*)- or (*R*)-1-PEDH (Table 2). In contrast, specific (*R*)-PEDH activities of 57.9 ± 2.9 and 18.5 ± 5.7 nmol min^{-1} (mg protein)$^{-1}$ were recorded in cells grown on acetophenone and phenol, respectively.

Table 2 Activities of (*R*)- and (*S*)-1-phenylethanol dehydrogenases (PEDH) in cell-free extracts of *A. aromaticum* grown under denitrifying conditions on different substrates

Growth substrate	Specific activity with	
	(*S*)-1-phenylethanol	(*R*)-1-phenylethanol
Benzoate	<1	<1
Acetophenone	5.1 ± 0.8	57.9 ± 2.9
Ethylbenzene	21.8 ± 1.0	16.1 ± 0.9
p-Ethylphenol	83.6 ± 3.7	88.2 ± 11.8
Phenol	<1	18.5 ± 5.7

Specific activities are given in nmol per min and mg of protein. The values given are the average of at least three parallel measurements including the standard deviation

The highest specific activities of 83.6 ± 3.7 and 88.2 ± 11.8 nmol min^{-1} (mg protein)$^{-1}$ for (*S*)- and (*R*)-PEDH, respectively, were determined in *p*-ethylphenol-grown cells (Table 2). Since the predicted *eph* operon contains two genes coding for short-chain dehydrogenases (SDR family), namely *chnA* and *ebA309* (Fig. 1b), that had been identified as substrate-induced proteins (Rabus et al. 2005; Wöhlbrand et al. 2008; Rabus et al. 2014), the measured activities suggested that one of them may be (*S*)-specific, the other (*R*)-specific. To confirm this hypothesis, we overproduced both enzymes in *E. coli*, purified them using *Strep*-tag affinity chromatography and tested their activities with either (*R*)- or (*S*)-1-phenylethanol. The ChnA isoenzyme was indeed only active with (*R*)-phenylethanol with a specific activity of 3.0 U mg^{-1}, and no activity was found with (*S*)-phenylethanol. Purified *ebA309* gene product exhibited a specific activity of 4.2 U mg^{-1} with (*S*)-phenylethanol and was inactive with (*R*)-phenylethanol. Both enzymes also catalyzed the NADH-dependent reduction of the natural product *p*-hydroxyacetophenone, with specific activities of 1.4 and 1.0 U mg^{-1}, respectively.

Acetophenone and *p*-hydroxyacetophenone carboxylases

In '*A. aromaticum*' cells grown on ethylbenzene or acetophenone, the metabolic intermediate acetophenone is carboxylated to benzoylacetate by acetophenone carboxylase ApcABCDE (Jobst et al. 2010). In this study, we observed specific Apc activities of 4.3 and 7.5 nmol min^{-1} (mg protein)$^{-1}$ in the extracts of cells grown on ethylbenzene or acetophenone, respectively, while none of the other extracts showed any measurable activity (Table 3). Moreover, no activity was detected in cell-free extracts

Table 3 Activities of acetophenone and *p*-hydroxyacetophenone carboxylases in cell-free extracts of *A. aromaticum* grown under denitrifying conditions on different substrates

Growth substrate	Specific activity with	
	Acetophenone	*p*-Hydroxyacetophenone
Benzoate	<0.5	<0.5
Acetophenone	7.5 ± 0.5	<0.5
Ethylbenzene	4.3 ± 0.8	<0.5
p-Ethylphenol	<0.5	<0.5
Phenol	0.8 ± 1.1	0.7 ± 0.6

Specific activities are given in nmol [^{14} C]bicarbonate incorporated into acid-stable products per min and mg of protein. The values given are the average of at least three parallel measurements including the standard deviation

of ethylbenzene- or acetophenone-grown cells with *p*-hydroxyacetophenone as substrate.

The presence of Apc in ethylbenzene- and acetophenone-grown cells was visible from the protein patterns of cell extracts separated by SDS-PAGE (Fig. 2a), and has been confirmed by immunoblotting experiments with antiserum raised against the purified Apc core enzyme (Fig. 2c). The three large subunits of Apc (87, 75 and 70 kDa) were present in large amounts only in cells grown on ethylbenzene or acetophenone and were found to be specifically labeled in the immunoblots (Fig. 2c, lanes A and EB). The relative intensities of the respective Apc subunits in the immunoblots and the Coomassie stained gels appeared to be consistent with the 1.7-fold difference of the respective specific enzyme activities (Fig. 2a, lanes A and EB, Table 3). No immunoreactive polypeptides were detected in cells grown on benzoate or phenol, but interestingly, faint signals corresponding to the large subunits of Apc were seen in cells grown on *p*-ethylphenol in the immunoblot assay (Fig. 2c, lane EP).

The degradation pathway of *p*-ethylphenol is predicted to proceed via *p*-hydroxyacetophenone as intermediate (Wöhlbrand et al. 2008). Since this compound is not turned over by Apc (Table 3; Jobst et al. 2010), a biotin-dependent carboxylase (XccABC) encoded in the apparent *p*-ethylphenol-catabolic operon has been proposed to play a role in *p*-hydroxyacetophenone carboxylation, based on the proteomic detection of XccA and XccB (Wöhlbrand et al. 2008; Rabus et al. 2014). However, the predicted activity of XccABC as *p*-hydroxyacetophenone carboxylase could not be confirmed by an enzyme assay based on $^{14}CO_2$ incorporation (Table 3). To determine the presence of the predicted carboxylase, we stained blotted SDS gels for the biotin-containing XccB subunit with a commercially available horseradish peroxidase-avidin conjugate (Fig. 2e). We observed the presence of two constitutive biotin-containing proteins in every extract, which were identified via mass spectrometry as the alpha subunit of propionyl-CoA carboxylase (PccA; 73.1 kDa) and the biotin carboxyl carrier protein of acetyl-CoA carboxylase (AccB; 16 kDa). In addition, we detected an additional protein of 17.6 kDa exclusively in *p*-ethylphenol-grown cells (Fig. 2e, lane EP), which was identified via mass spectrometry as the XccB subunit of the expected biotin-dependent carboxylase encoded in the predicted operon (Fig. 1; Wöhlbrand et al. 2008). Two additional faint bands between XccB and AccB, which were only detected in *p*-ethylphenol-grown cells, are probably degradation products of XccB (Fig. 2e, lane EP).

Benzoylacetate-CoA ligase and other CoA ligases

Anaerobic ethylbenzene degradation proceeds via benzoylacetate as intermediate (Rabus and Heider 1998), which is believed to be activated to the coenzyme A (CoA) thioester by a specific benzoylacetate-CoA ligase (Bal) encoded in the *apc–bal* operon (Rabus et al. 2002). Therefore, we investigated '*A. aromaticum*' cells grown on different substrates for the presence of the corresponding enzyme. The standard assay for CoA ligases could not be used because we observed a direct NADH-dependent reduction activity of benzoylacetate, which obscured the output of the coupled enzyme assay (see Materials and methods, data not shown). A photometric assay based on the NADH-dependent reduction of benzoylacetate yielded indeed quite significant benzoylacetate reduction activities in all extracts with the highest values recorded in *p*-ethylphenol-grown cells (Table 4). We assume that this represents a side activity of one or more alcohol dehydrogenases present in the extracts.

Therefore, we present an alternative assay to evaluate Bal activity based on the increased UV absorbance of the respective CoA thioester at 290–310 nm under slightly alkaline conditions in the presence of Mg^{2+} (Decker 1959). As a control, benzoate-CoA ligase (BclA) activities were determined by the same assay. The absorption coefficient at 300 nm was experimentally determined by converting limiting known concentrations of either CoA or benzoate to completion ($\varepsilon = 2.08\ mM^{-1}\ cm^{-1}$). The highest specific Bal activities of 10 ± 1 nmol min^{-1} (mg of protein)$^{-1}$ were observed in cells grown on ethylbenzene or acetophenone (Table 4), which is consistent with the observed growth rates (data not shown). The enzyme was not detectable in benzoate-grown cells, but low specific activities were recorded in *p*-ethylphenol- and phenol-grown cells (Table 4).

To assure that the cell extracts used were in proper physiological conditions, the activities of benzoate- and succinate-CoA ligase were assayed by the standard NADH-coupled assay (Ziegler et al. 1987; Schühle et al. 2003), because all investigated metabolic pathways involve

Table 4 Activities of different CoA ligases and an apparent benzoylacetate reductase of *A. aromaticum* grown under denitrifying conditions on different substrates

Growth substrate	Direct photometric assay		Indirect photometric assay		
	Benzoate-CoA ligase	Benzoylacetate-CoA ligase	Benzoylacetate reductase	Benzoate-CoA ligase	Succinate-CoA ligase (ADP-forming)
Benzoate	90 ± 1	<1	14 ± 3	80 ± 5	336 ± 19
Acetophenone	158 ± 2	10 ± 1	8 ± 2	100 ± 12	574 ± 26
Ethylbenzene	28 ± 1	10 ± 1	7 ± 2	17 ± 2	885 ± 60
p-Ethylphenol	143 ± 5	5 ± 2	27 ± 1	126 ± 9	352 ± 38
Phenol	19 ± 1	3 ± 1	2 ± 4	9 ± 1	412 ± 18

Specific activities are given in nmol per min and mg of protein. The values given are the average of at least three parallel measurements including the standard deviation

benzoyl-CoA and succinyl-CoA as intermediates and the enzymes are known to be generally induced during anaerobic degradation of any aromatic compound in various bacteria (Harwood et al. 1998; Carmona et al. 2009; Fuchs et al. 2011). Relatively high succinate-CoA ligase activities were detected in all extracts tested in this study (Table 4). The values of the specific activities recorded by the direct and the coupled photometric BclA assays were in good agreement in the respective cell extracts, valuating the A_{300}-based assay (Table 4).

Discussion

In this study, we analyzed the presence of key enzymes involved in anaerobic ethylbenzene and *p*-ethylphenol degradation to the common metabolic intermediate benzoyl-CoA in '*Aromatoleum aromaticum*' strain EbN1 (Fig. 1). By combining various methods, we show that distinct and specifically induced enzymes are used during these two metabolic pathways and establish assays to detect their presence.

Enzymes of ethylbenzene degradation

EbDH, catalyzing the initial step of ethylbenzene metabolism, was exclusively induced in ethylbenzene-degrading cells, consistent with previous observations (Kniemeyer and Heider 2001a). This was especially substantiated by immunoblotting data with antiserum against EbDH, but also by recording activities for the hydroxylation of its hydrocarbon-type substrates ethylbenzene and indane. These activities appear to be highly specific for EbDH, whereas alternative substrates of EbDH, e.g., *p*-ethylphenol, appear to be hydroxylated also by other enzymes such as EpMH. The complete absence of either enzyme activity or immunologically detectable subunits of EbDH in any other extract also confirms the previously observed strictly sequential regulation of the enzymes of ethylbenzene and acetophenone degradation (Kühner et al. 2005).

The second enzyme of ethylbenzene metabolism is an (*S*)-specific 1-phenylethanol dehydrogenase encoded in a common operon with the genes for EbDH (Rabus et al. 2002) and therefore expected to be coinduced in ethylbenzene-grown cells. The activities for (*S*)-1-phenylethanol in ethylbenzene-grown cells were slightly higher than those recorded for (*R*)-1-phenylethanol, and both activities varied by about 20 % in independently grown cells (data not shown). This variation may be explained with the need to detoxify excess cytoplasmic acetophenone by additional alcohol dehydrogenase isoenzymes either to (*S*)- or (*R*)-1-phenylethanol and to different extents in independent cultures (Champion et al. 1999; Kniemeyer and Heider 2001b; Höffken et al. 2006; Dudzik et al. 2014). This notion is also corroborated by the observed presence of PEDH isoenzymes of both enantiospecificities in acetophenone-grown cells, which do no longer produce the ethylbenzene-induced (*S*)-specific enzyme encoded in the *ebd–ped* operon (Rabus et al. 2002). Likewise, the observed specific (*R*)-PEDH activity in cells grown on phenol may also be related to detoxifying functions. As '*A. aromaticum*' strain EbN1 contains 32 genes for SDR-type alcohol dehydrogenases in its genome (Rabus et al. 2005), it will not be easy to identify the actual enzymes responsible for these apparent detoxification functions.

We confirmed the reported specificity of purified acetophenone carboxylase (ApcABCDE) toward its natural substrate (Jobst et al. 2010), since no activity was detected in cell-free extracts of ethylbenzene- or acetophenone-grown cells with *p*-hydroxyacetophenone as substrate. Additionally, we substantiate the presence of Apc in both ethylbenzene- and acetophenone-grown cells, based on immunological detection with antiserum against the purified enzyme. A surprising result was the presence of low amounts of immunoreactive Apc subunits in *p*-ethylphenol-grown cells, even if no enzyme activity was measurable. We

assume that Apc activity in these cells was below the detection limit of the assay, which was determined at around 10 % of the activity observed in ethylbenzene-grown cells (Table 3). This suggests a possible low-level cross-induction of the *apc–bal* operon in *p*-ethylphenol-degrading cells that is accessible via immunological detection, but not via enzyme assays.

The activity of an apparent benzoylacetate-CoA ligase (Bal) was detected in extracts of ethylbenzene- and acetophenone-grown cells, but was absent in those of benzoate-grown cells. This enzyme activity is probably correlated with the induced expression of the *bal* gene as part of the *apc–bal* operon.

Although the detected values of some specific activities of the investigated enzymes were lower than those reported in previous studies, they are sufficient to explain the growth rates of the respective cultures and are in line with the activity range observed in previous experiments in different cell batches (Kniemeyer and Heider 2001a, b; Szaleniec et al. 2003, 2007; Jobst et al. 2010; Knack et al. 2012 and unpublished data) or (for CoA ligases) as shown in analogous studies with cell-free extracts of *Thauera aromatica* (Heider et al. 1998; Schühle et al. 2003).

Enzymes of *p*-ethylphenol degradation

The results determined in this study substantiate the predicted *p*-ethylphenol degradation pathway by assaying some of the enzymes involved, including the presence and specificity of a heme *c*-containing *p*-ethylphenol-hydroxylating enzyme (EpMH) distinct from EbDH in '*A. aromaticum*' cells degrading *p*-ethylphenol. EpMH appears to hydroxylate only *p*-ethylphenol and related phenolic analogs such as 5′-hydroxyindane, but no hydrocarbons such as ethylbenzene or indane. EpMH was highly induced in *p*-ethylphenol-grown cells, while background activity levels recorded in control cells (grown on benzoate or phenol) may be attributed to unspecific cross-induction of the paralogous *p*-cresol methylhydroxylase isoenzyme. The slightly increased activity determined in ethylbenzene-grown cells may be explained by unspecific turnover of *p*-ethylphenol by EbDH as mentioned above and reported for the purified enzyme (Knack et al. 2012). We established an enzyme assay coupled with the ferricenium ion instead of PMS/DCPIP as electron acceptor, which apparently yields higher and more reliable activity values of EpMH. These observations suggest that ferricenium should be used as the preferred electron acceptor for obtaining higher and more selective activity readings for either EpMH or EbDH. The apparent necessity of a *p*-hydroxy group in the substrates also fits nicely with the proposed reaction mechanism of this type of hydroxylases by involving quinone-methide or quinone-ethide intermediates (Heider and Fuchs 1997b). As reported for EpMH from *P. putida*, the enzyme is expected to catalyze *p*-ethylphenol hydroxylation preferentially to the level of the alcohol with only a small amount of *p*-hydroxyacetophenone generated in a subsequent hydroxylation reaction (Reeve et al. 1990), in contrast to the generally occurring double hydroxylation reactions reported for *p*-cresol methylhydroxylase, which oxidizes *p*-cresol to *p*-hydroxybenzaldehyde (McIntire et al. 1985; Rudolphi et al. 1991; Carmona et al. 2009). Although EpMH of *P. putida* has been shown to produce (*R*)-1-(*p*-hydroxyphenyl) ethanol (Reeve et al. 1990), the enantiospecificity of the EpMH isoenzyme from '*A. aromaticum*' needs yet to be shown.

The presence of coinduced genes coding for alcohol dehydrogenases in the *eph* operon is consistent with the expected activity of EpMH in preferentially converting *p*-ethylphenol to the alcohol, but not further to the ketone (Reeve et al. 1990; Wöhlbrand et al. 2008; Carmona et al. 2009). The activities of both (*R*)- and (*S*)-specific aromatic alcohol dehydrogenase appear to be strongly induced in *p*-ethylphenol-grown cells, consistent with the identified activities of the overproduced *chnA* and *ebA309* gene products as (*R*)- and (*S*)-PEDHs, respectively. As known for the ethylbenzene-induced isoenzyme encoded by the *ped* gene (33 and 36 % identity, respectively; Kniemeyer and Heider 2001b; Rabus et al. 2002; Höffken et al. 2006; Wöhlbrand et al. 2008; Rabus et al. 2014), these enzymes seem to be equally active with the respective enantiomers of either 1-phenylethanol or 1-(*p*-hydroxyphenyl)ethanol. This is also consistent with the high recorded activities of both purified enzymes for either 1-phenylethanol oxidation or *p*-hydroxyacetophenone reduction. It is still an open question why two enzymes of different enantiospecificity are present, which are apparently induced to about equal activities. As outlined for acetophenone metabolism, they may have detoxifying functions for the ketone intermediate or serve some other, yet-unknown function in the further degradation pathway.

The proposed biotin-dependent carboxylase (XccABC; Wöhlbrand et al. 2008; Rabus et al. 2014) is distinct from other catabolic carboxylases in '*A. aromaticum*' such as Apc (Jobst et al. 2010), acetone carboxylase (Schühle and Heider 2012) or phenylphosphate carboxylase (Schmeling and Fuchs 2009). We assume that the genes coding for the three subunits of the predicted biotin-dependent carboxylase (XccABC) are indeed involved in carboxylation of *p*-hydroxyacetophenone (or potentially another intermediate generated from *p*-ethylphenol during the degradation pathway), since the substrate-specific induction of the XccB subunit was clearly identified by biotin-blotting and mass spectrometry. The predicted activity of a *p*-hydroxyacetophenone-carboxylating biotin-dependent enzyme complex was not measurable via the enzyme assay used for

Apc. This suggests that XccABC may need yet-unknown supplements or may act on a different substrate than the ketone to show activity. In contrast, Apc does not appear to be involved in anaerobic *p*-ethylphenol metabolism.

After the still elusive carboxylation reaction of *p*-hydroxyacetophenone to the 3-oxoacid *p*-hydroxybenzoylacetate, the CoA ligase AcsA encoded in the *eph* operon is expected to activate the product to the CoA thioester (Wöhlbrand et al. 2008). The observed low activity of a CoA ligase activating the substrate analog benzoylacetate in extracts of *p*-ethylphenol-grown cells may correspond to this enzyme, although similar activities were also measured in phenol-grown cells, which should not have an analogous enzyme induced. At this point it cannot be ruled out that both observed activities in *p*-ethylphenol- and phenol-grown cells are due to the cross-reactivity of unrelated CoA ligases.

In conclusion, this study shows the (almost) exclusive presence and specificity of the key enzymes involved in the respective degradation pathway of the chemically very similar carbon sources ethylbenzene and *p*-ethylphenol. Additionally, the combined results provide evidence to substantiate the *p*-ethylphenol catabolic pathway predicted on the basis of a proteomic analysis and should serve as basis for future studies on their biochemistry and regulation. The presented methods should allow assaying the intrinsic physiological state of cells growing on substrate mixtures and the mechanisms behind the substrate-specific induction as well as the discrimination mechanisms of these two pathways.

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