Molecular Genetic and Crystal Structural Analysis of 1-(4-Hydroxyphenyl)-Ethanol Dehydrogenase from ‘Aromatoleum aromaticum’ EbN1

Imke Büsing\textsuperscript{a} H. Wolfgang Höffken\textsuperscript{b} Michael Breuer\textsuperscript{c} Lars Wöhlbrand\textsuperscript{a} Bernhard Hauer\textsuperscript{c} Ralf Rabus\textsuperscript{a, d}

\textsuperscript{a}Institute for Chemistry and Biology of the Marine Environment (ICBM), Carl von Ossietzky University Oldenburg, Oldenburg, \textsuperscript{b}Computational Chemistry and Biology, and \textsuperscript{c}White Biotechnology Research, BASF SE, Ludwigshafen, and \textsuperscript{d}Max Planck Institute for Marine Microbiology, Bremen, Germany

\textbf{Abstract}

The dehydrogenation of 1-(4-hydroxyphenyl)-ethanol to 4-hydroxyacetophenone represents the second reaction step during anaerobic degradation of 4-ethylphenol in the denitrifying bacterium ‘Aromatoleum aromaticum’ EbN1. Previous proteogenomic studies identified two different proteins (ChnA and EbA309) as possible candidates for catalyzing this reaction [Wöhlbrand et al: J Bacteriol 2008; 190: 5699–5709]. Physiological-molecular characterization of newly generated unmarked \textit{in-frame} deletion and complementation mutants allowed defining ChnA (renamed here as Hped) as the enzyme responsible for 1-(4-hydroxyphenyl)-ethanol oxidation. Hped [1-(4-hydroxyphenyl)-ethanol dehydrogenase] belongs to the ‘classical’ family within the short-chain alcohol dehydrogenase/reductase (SDR) superfamily. Hped was overproduced in \textit{Escherichia coli}, purified and crystallized. The X-ray structures of the apo- and NAD\textsuperscript{+}-soaked form were resolved at 1.5 and 1.1 Å, respectively, and revealed Hped as a typical homotetrameric SDR. Modeling of the substrate 4-hydroxyacetophenone (reductive direction of Hped) into the active site revealed the structural determinants of the strict (\textit{R})-specificity of Hped (Phe\textsuperscript{187}), contrasting the (\textit{S})-specificity of previously reported 1-phenyl-ethanol dehydrogenase (Ped; Tyr\textsuperscript{93}) from strain EbN1 [Höffken et al: Biochemistry 2006;45:82–93].

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\textbf{Key Words}

Anaerobic p-ethylphenol degradation · 1-(4-Hydroxyphenyl)-ethanol dehydrogenase · \textit{In-frame} deletion mutation · Proteogenomics · Crystal structure · Enantioselectivity · Denitrifier · ‘Aromatoleum aromaticum’ EbN1

\textbf{Introduction}

Aromatic compounds are structurally diverse and energy-rich compounds that challenge biodegradation with their chemical stability. Under oxic conditions (presence of O\textsubscript{2}), microorganisms employ highly reactive oxygen species (O\textsubscript{2}-derived) in oxygenase catalyzed reactions for compound activation and ring cleavage [e.g. Perez-Pantoja et al., 2012]. Under anoxic conditions (absence of O\textsubscript{2}), which dominate in many habitats such as marine and freshwater sediments as well as deeper soil layers, a large dedicated to Fritz Widdel on occasion of his 65th birthday. I.B. and H.W.H. contributed equally to this work.
diversity of (facultative) anaerobic bacteria occurs [Kaser and Coates, 2010; Widdel and Rabus, 2001; Widdel et al., 2010]. They have evolved a broad spectrum of intriguing reactions for substrate activation and dearromatization [Carmona et al., 2009; Fuchs et al., 2011; Gibson and Harwood, 2002; Widdel and Musat, 2010]. The present study organism, 'Aromatoleum aromaticum' EbN1 [Rabus and Widdel, 1995], is affiliated with the betaproteobacterial 'Aromatoleum'/Azoarcus/Thauera cluster, harboring a large fraction of the currently known anaerobic degraders of aromatic compounds [Widdel et al., 2010]. On the basis of its genome [Rabus et al., 2005], comprehensive physiological-proteomic experiments allowed the discovery of several new catabolic capacities and establishing 'A. aromaticum' EbN1 as a model organism to study anaerobic aromatic compound degradation on the systems biology level [Rabus et al., 2014; Wöhlbrand et al., 2007].

\( p \)-Ethylphenol enters the environment from anthropogenic as well as natural sources (see references cited in Wöhlbrand et al. [2008]). The anaerobic degradation of this phenolic compound was for the first time elucidated in 'A. aromaticum' EbN1, using differential proteogenomic and metabolite analysis [Wöhlbrand et al., 2008]. Notably, the initial activation and transformation of the ethyl side chain resembles that of ethylbenzene [Jobst et al., 2010; Kniemeyer and Heider, 2001a; Rabus et al., 2002], even though a completely different set of enzymes is involved: \( p \)-ethylphenol is initially hydroxylated to 1-(4-hydroxyphenyl)-ethanol, which is subsequently dehydrogenated to 4-hydroxyacetophenone. The latter is presumably transformed to the central intermediate benzoyl-CoA via carboxylation, thiolytic removal of acetyl-CoA and reductive dehydroxylation (fig. 1a). All involved genes colocalize on the chromosome in an operon-like structure and most encoded proteins were specifically formed and identified in \( p \)-ethylphenol-adapted cells of 'A. aromaticum' EbN1 [Wöhlbrand et al., 2008].

With respect to the second reaction step in the anaerobic \( p \)-ethylphenol degradation pathway, the current proteogenomic evidence is ambiguous. Two dehydrogenases (EbA309, predicted alcohol dehydrogenase; ChnA, predicted cyclohexanol dehydrogenase) could catalyze an NAD\(^+\)-dependent dehydrogenation of 1-(4-hydroxyphenyl)-ethanol to 4-hydroxyacetophenone. Moreover, the stereochemistry of the dehydrogenation reaction at the chiral carbon atom of 1-(4-hydroxyphenyl)-ethanol is unclear at present. Notably, the analogous reaction in the anaerobic ethylbenzene degradation pathway, catalyzed by NAD\(^+\)-dependent 1-phenylethanol dehydrogenase (Ped), is (S)-specific [Höffken et al., 2006; Kniemeyer and Heider, 2001b]. Other known 1-phenylethanol-convert-
1-(4-Hydroxyphenyl)-Ethanol DH of Strain EbN1

**Accession** | **Protein** | **Predicted function** | **Mascot Score**
---|---|---|---
**wt** | **hped/ebA309- compl.** | **hped- compl.** | **ebA309- compl.**
---|---|---|---
ebA299 | PchC | Probable p-ethylphenol methylenehydroxylase subunit | 118.3 | 155.5 | 210.7 | 163.7 | 173.7
ebA300 | PchF | Probable p-ethylphenol methylenehydroxylase subunit | 754.6 | 814.7 | 570.0 | 756.7 | 392.8
ebA303 | EbaA503 | Alcohol dehydrogenase | 291.7 | 335.8 | 209.4 | 184.9 | 99.4
ebA305 | EbaA305 | Conserved hypothetical protein | 239.9 | 118.4 | 107.2 | 126.8 | 100.4
ebA306 | TioL | Predicted thiolase | 516.2 | 555.2 | 293.9 | 255.9 | 118.5
ebA307 | Hped | Hydroxy-phenylethanol dehydrogenase | 460.2 | 0.0 | 67.6 | 112.2 | 0.0
ebA309 | EbaA309 | Putative dehydrogenase | 516.2 | 0.0 | 66.6 | 112.3 | 0.0
ebA310 | EbaA10 | FAD linked oxidase | 584.0 | 722.3 | 415.9 | 417.5 | 333.5
ebA312 | EbaA12 | Predicted sugar phosphatases of the HAD superfamily | 234.6 | 287.0 | 144.6 | 57.6 | 150.1
ebA314 | XccA | Putative carboxylase subunit of acetyl-CoA carboxylase-like enzyme | 407.4 | 571.8 | 167.0 | 172.8 | 243.5
ebA316 | XccC | Biotin carboxylase subunit of acetyl-CoA carboxylase-like enzyme | 637.2 | 275.2 | 191.0 | 252.8 | 190.0
ebA318 | EbaA318 | Conserved hypothetical protein | 449.9 | 539.4 | 381.6 | 185.8 | 199.2

**Legend**
- wt = wild type
- compl. = complemented mutant
- Δ = Δhped/ebA309

**Diagram a**
- p-ethylphenol
- 1-(4-Hydroxyphenyl)-ethanol
- 4-Hydroxycetophenone
- 4-Hydroxybenzoyl-CoA
- Benzoyl-CoA

**Diagram b**
- Wild type
- Δhped/ebA309
- tioL
- ebA302
- ebA303
- ebA310
- ebA312
- tbioL
- ebA309
- 1 kb
- tioL/ebA310 wt
- 350 bp
- hped
- 331 bp
- ebA309
- 320 bp
- ebA310
- 366 bp

**Diagram c**
- Size (bp)
- 200000
- 100000
- 50000
- 10000

**Diagram d**
- wt - wild type
- compl. - complemented mutant
ing dehydrogenases are either (S)-specific and Zn^{2+}-dependent (*Rhodococcus erythropolis* [Aboktise and Hummel, 2003]) or (R)-specific and NADP^{+}-dependent (*Lactobacillus brevis* [Niefind et al., 2003]). In general, such enantioselective NAD(P)^{+}-dependent dehydrogenases operating in reductive direction are valuable catalysts for the biotechnological synthesis of chiral compounds [Hummel, 1999].

In the present study, application of molecular genetics (unmarked in-frame deletion and in trans expression) allowed identifying ChnA (renamed here as Hped) as the enzyme responsible for 1-(4-hydroxyphenyl)-ethanol oxidation in *A. aromaticum* EbN1. Moreover, analysis of the Hped crystal structure resolved the (R)-specificity of the dehydrogenation reaction.

**Results and Discussion**

To avoid ambiguous assignments of gene names, from here on hped for 1-(4-hydroxyphenyl)-ethanol dehydrogenase is used instead of the original designation *chnA*, which had been solely based on product homology to described cyclohexanone dehydrogenase [Rabus et al., 2005].

*Generation and Molecular Characterization of an Unmarked hped/ebA309 In-Frame Deletion and Its Complementation Mutants*

The second reaction of the anaerobic *p*-ethylphenol degradation pathway in *A. aromaticum* EbN1 may be performed by two predicted dehydrogenases, Hped and EbA309, adjacently encoded within the *p*-ethylphenol gene cluster (fig. 1a). Since both proteins are specifically formed during anaerobic growth of strain EbN1 with *p*-ethylphenol and 4-hydroxyacetophenone, respectively, it was unclear which enzyme catalyzes the oxidation of 1-(4-hydroxyphenyl)-ethanol to 4-hydroxyacetophenone [Wöhlbrand et al., 2008]. To determine the catalytically active enzyme, an unmarked in-frame deletion mutant (Δhped/ebA309) was generated by means of homologous recombination. Notably, duplicating the size of the homologous region in the knockout vector pronouncedly increased the knockout efficiency (∼50%) as compared to previous experiments (<4% [Wöhlbrand and Rabus, 2009]). The mutant genotype harbors only the start codon of *hped* and the stop codon of *ebA309* separated by a *PstI* restriction site, maintaining the reading frame. Accordingly, neither *hped* nor *ebA309* could be amplified by PCR from genomic DNA of the mutant strain using gene-specific primers. In addition, primers located in the 5’- and 3’-neighboring genes *ebA310* and *tioL*, respectively, yielded a small 350-bp mutant-specific product instead of a 2,000-bp wild-type amplicon (fig. 1b, c). The presence of polar effects caused by the deletion can be excluded since transcripts of the neighboring genes *ebA310* and *tioL* were detected in the mutant growing with 4-hydroxyacetophenone (fig. 1d). Furthermore, 5 of the 6 proteins encoded downstream of *hped*, as well as 5 of the 7 proteins encoded upstream of *ebA309*, were identified in these cells (fig. 1e).

In order to restore the wild-type phenotype and to identify the enzyme catalyzing the dehydrogenation of 1-(4-hydroxyphenyl)-ethanol, three complementation mutants were generated: a hped/ebA309-complemented mutant (genotype: Δhped/ebA309, pBBRMCS-4 Ω*hped/ebA309*), a hped-complemented mutant (genotype: Δhped/ebA309, pBBRMCS-4 Ω*hped*) and a ebA309-complemented mutant (genotype: Δhped/ebA309, pBBRMCS-4 Ω*ebA309*). The complemented genes were constitutively expressed from the broad host range plasmid pBBRMCS-4 as the presence of their transcripts and proteins in the respective strains could be confirmed by transcript and proteomic analysis (fig. 1d, e).

*Identification of Hped as Productive Dehydrogenase for 1-(4-Hydroxyphenyl)-Ethanol Oxidation*

The generated deletion mutant Δhped/ebA309 revealed no growth with *p*-ethylphenol after 80 h of incubation, while the wild-type strain reached the stationary growth phase already after 45 h. Interestingly, the hped/ebA309-complemented and hped-complemented mutant strains restored growth with *p*-ethylphenol after a prolonged lag-phase, though with decreased maximum growth rate and yield (fig. 2a). The latter may be due to the *p*-ethylphenol-evoked solvent stress in combination with the applied ampicillin (for plasmid maintenance). Notably, in trans expression of *ebA309* did not restore anaerobic growth with *p*-ethylphenol, suggesting Hped to be the only catalytically active dehydrogenase for the investigated reaction. In contrast to *p*-ethylphenol, growth with 4-hydroxyacetophenone was unaffected by the gene deletion, as Hped and EbA309 are not required for degradation of this intermediate of the anaerobic *p*-ethylphenol degradation pathway (fig. 2b). The hped-complemented mutant strain revealed a prolonged lag-phase, while maximal growth rate and optical density similar to the wild-type strain EbN1 were obtained. To exclude any secondary effects caused by the gene deletion, growth with all known substrates of strain EbN1 other than *p*...
ethylphenol was tested with the Δhped/ebA309 mutant, revealing no differences to the wild type. This molecular genetic evidence for the hped encoded dehydrogenase to be solely responsible for 1-(4-hydroxyphenyl)-ethanol oxidation to 4-hydroxyacetophenone in 'A. aromaticum' EbN1 motivated the continuative enzymatic analysis with overproduced Hped in Escherichia coli extracts (see Preliminary Enzymatic Characterization of Hped vs. Ped, as well as fig. S2 and S3 in the supplementary material; see www.karger.com/doi/10.1159/000439113 for all suppl. material) and structural studies with the purified and crystallized Hped protein.

Enantioselectivity
EbA309 has opposite enantioselectivity for acetophenone derivatives in comparison to Hped. Acetophenone, for example, is converted by Hped almost exclusively (ee: >99.7%) to the respective (1R)-1-phenylethanol, whereas EbA309 gives rise to (1S)-1-phenylethanol (ee: >99.7%). In accordance with Prelog [1964], we can safely assign Hped as a Prelog-dehydrogenase contrary to EbA309, which is an anti-Prelog-dehydrogenase.

The Overall Protein Structure
The structure of Hped was solved as apo- and holo-form with a resolution of 1.5 and 1.1 Å, respectively. The quality of the structures is reflected by the good R-factors and excellent stereochemical parameters (online suppl. table 1). Hped is a typical member of the short-chain alcohol dehydrogenase/reductase (SDR) superfamily with a classical Rossmann fold structure. The central twisted parallel β-sheet with seven strands is flanked by six α-helices, three on each side. Two additional short helices (αFG1 and αFG2) between strand βF and helix αG form the substrate-binding site (fig. 3a–c). There are two monomers of 248 amino acid residues each in the crystallographic asymmetric unit.

Sequence and Structural Features Compared with Other SDRs
Hped belongs to the large SDR superfamily, members of which have been intensively studied with respect to structure, catalytic mechanism and evolution (e.g. [Jörnvall et al., 1981, 2010; Ladenstein et al., 2008]). According to Kallberg et al. [2002], Hped belongs to the cD1d-subfamily of the ‘classical’ family within the SDR superfamily. Comparison to sequences of other SDRs shows, with the exception of one, that all highly conserved and structurally relevant amino acids as tabulated by Oppermann et al. [2003] and Kallberg et al. [2002] are also present in Hped (fig. 3d), and are discussed in the following paragraphs.

The first motif is the TGXGXG sequence. The Oγ Thr11 forms a hydrogen bond to the main chain nitrogen of Asn89, stabilizing the ends of strands βA and βD of the central β-sheet. At position 12, a larger amino acid than...
glycine would lead to a clash with Ala\textsuperscript{90} and also interfere with the cofactor binding. Gly\textsuperscript{16} and Gly\textsuperscript{18} are located at the N-terminal end of helix \( \alpha B \). A larger amino acid at position 16 would clash with the phosphates of the NAD\textsuperscript{+} cofactor. A larger amino acid at position 18 would clash with the carbonyl oxygen of Gly\textsuperscript{12} and ND2 Asn\textsuperscript{89}.

The Asp\textsuperscript{60} in the bacterial 3β/17β-hydroxysteroid dehydrogenase is characterized by Oppermann et al. [2003] as stabilizing the adenine ring pocket with weak interaction to the adenine. The equivalent Asp\textsuperscript{62} in Hped has the same role. The oxygen OD1 Asp\textsuperscript{62} forms a hydrogen bond to N Ala\textsuperscript{64} and stabilizes the loop connecting the \( \beta \)-strand C and helix \( \alpha D \). The OD1 Asp\textsuperscript{62} also forms a hydrogen bond to the adenine nitrogen N6A with a distance of 2.96 Å.

The NNAG motif shows different conformations in the apo- and holo-form (see Further Details on the Structure of Hped in the supplementary material).

The four amino acids Asn\textsuperscript{114}, Ser\textsuperscript{142}, Tyr\textsuperscript{155} and Lys\textsuperscript{159} form the catalytic tetrad. For further details see Structural Insights into the Catalytic Mechanism of Hped below.

The side chain amino group of Asn\textsuperscript{181} (Asn\textsuperscript{179} according to Oppermann et al. [2003]) is involved in a tight network of hydrogen bonds. The oxygen OD1 Asn\textsuperscript{181} is hydrogen bonded to the main chain nitrogen of Ser\textsuperscript{237} (3.0 Å) and also makes a weak CH-O hydrogen bond to the \( \alpha \)-atom of Thr\textsuperscript{235} (3.18 Å). The nitrogen ND2 Asn\textsuperscript{181} is hydrogen bonded to the main chain oxygen of Val\textsuperscript{234} (2.98 Å) and to a water molecule (No. 2169) with a distance of 2.88 Å. This water molecule is further hydrogen bonded to the carbonyl oxygen atoms of Leu\textsuperscript{226} (3.13 Å) and Ile\textsuperscript{37} (2.88 Å). This presumably reinforces the core of the monomer structure by connecting strands \( \beta F \) and \( \beta E \), helix \( \alpha G \), and the loop preceding helix \( \alpha G \), as also found in other ‘classical’ SDRs. The central role of Asn\textsuperscript{181} for the structural integrity of Hped is further evidenced by the close spatial proximity to Phe\textsuperscript{233} (see Further Structural Properties of Hped below).

The proline of the PG motif (Pro\textsuperscript{185} in Hped), which is very highly conserved within the ‘classical’ family among SDRs, is located at the back of the nicotinamide ring of NAD\textsuperscript{+} and seems to stabilize the active site. In Hped, the glycine of the PG motif is replaced by Ala\textsuperscript{186}, which reduces the flexibility of this part of the chain at the beginning of the substrate-binding loop. Ala\textsuperscript{186} is followed by Phe\textsuperscript{187}, which confines the substrate-binding pocket to assure the (\( R \))-stereospecificity of the (de)hydrogenation reaction. Like in many other SDRs, Thr\textsuperscript{190} forms a hydrogen bond to the carboxamide of the nicotinamide ring (OG1–N7N, 3.05 Å).

**Further Structural Properties of Hped**

The crystallographic two-fold axis generates the biologically active homotetramer with two types of hydrophobic dimerization interfaces and a 222 symmetry, as seen for many SDRs (fig. 3c). Phe\textsuperscript{233} plays a major role in the interaction between the monomers. It protrudes into the adjacent chain (monomer) and interacts with the two substrate-binding loops of the other chain (online suppl. fig. 4). Notably, Phe\textsuperscript{234} in Ped (analogous to Phe\textsuperscript{233} in Hped) is localized in the same spatial arrangement between two different chains. Phe\textsuperscript{234} was implicated in cooperativity of Ped [Höffken et al., 2006], which might also be the case with Phe\textsuperscript{233} and Hped. The hydrogen bonding of NAD\textsuperscript{+} to the N-terminus of helix \( \alpha B \) via O1–N Gly\textsuperscript{18} (3.13 Å) and O2–N Ile\textsuperscript{17} (2.87 Å) is displayed in online supplementary figure 5. A comparison of the Hped structure with NAD\textsuperscript{+} binding with those of Ped [Höffken et al., 2006], LVR [Sogabe et al., 2003] and FabG [Javidpour et al., 2014] is provided in online supplementary figures 6 and 7.

Additional information on the homodimer and tetramer, additional ligands, comparison of A and B chain, the

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**Fig. 3.** Overall structure of (1R)-1-(4-hydroxyphenyl)-ethanol dehydrogenase (Hped) from ‘A. aromaticum’ EbN1. **a** Structural overview of the Hped monomer with \( \alpha \)-helices highlighted in red and \( \beta \)-strands in blue. The shown NAD\textsuperscript{+}-cofactor is modeled into the structure. The labeling of secondary structure elements is according to Ghosh et al. [1991]. **b** Schematic diagram of secondary structure elements and their consecutive arrangement. **c** Quaternary structure of the tetramer. Subunits A, A’, B and B’ are indicated by letters and colored blue, yellow, brown and green (color in online version only). The arrows represent the symmetry axes, the R-axis is perpendicular to the paper. **d** Primary sequence of Hped highlighting localization of secondary structure elements, amino acids and motifs conserved among SDRs (bold, conserved amino acids; underlined, functionally defined amino acids according to Oppermann et al. [2003]; grey shading, motifs as defined by Kallberg et al. [2002] and catalytically relevant amino acids; green, NAD(H) binding; red, catalytic tetrad; white on blue background, steric forcing).
1-(4-Hydroxyphenyl)-Ethanol DH of Strain EbN1

A color version of the figure is available online.

MLLEGKTA VLAGNIGTIALTYAEGANVVSDIDSEWGETLALIEGKGGKAVFQHADTAHPEHDHELIAAKRAFGRILIDACNNAGISGEFTPTA

ETTDAGWQRVGILSLGVFGVRAQIRAGMLETCCGA1VNISFRAAQIGIAGITPGTAAGTVGVGLTKTVAMEYGSKCRINSVPLAGRTILT

RRQLEQMHMRRLRGLGETEEVLAWLSSDKASFVTGSYYAVDGGLAR
apo- and holo-structures as well as the comparison of apo- and holo-structures of Hped is provided in the section Further Details on the Structure of Hped in the online supplementary material and online supplementary table 2.

**Structural Insights into the Catalytic Mechanism of Hped**

The catalytic site of Hped reflects the well-known arrangement of the catalytic residues seen in almost all SDRs (fig. 4). Tyr\(^ {155} \) functions as the catalytic base, while Ser\(^ {142} \) helps to orient the substrate in an optimal position for hydride transfer. An acetate ion (see Further Details on the Structure of Hped – The Holo-Structure in the online suppl. material) is located in a position where the substrate carbonyl group is supposed to be positioned for hydride transfer. One oxygen atom of the acetate is hydrogen bonded to OH Tyr\(^ {155} \) (2.55 Å) and to OG Ser\(^ {142} \) (2.61 Å). This supports the role of Ser\(^ {142} \) in stabilizing the correct orientation. The methyl group of the acetate points into a hydrophobic small pocket, which is responsible for the Prelog specificity of Hped. Lys\(^ {159} \) interacts with the nicotinamide ribose and lowers the pK\(_{a}\) of the OH Tyr\(^ {155} \) [Oppermann et al., 2003]. Asn\(^ {114} \) is supposed to be part of the proton relay system. The main chain carbonyl oxygen forms a hydrogen bond to a highly conserved water molecule, which in turn is hydrogen bonded to the catalytic Lys\(^ {159} \). The side chain conformation of Asn\(^ {114} \) differs in the apo- and holo-form. Reduction of 4-hydroxyacetophenone should involve a nucleophilic attack of the pro-S hydride donated by NADH. Hydride transfer from nicotinamide occurs stereoselectively at the re side rather than the si side of 4-hydroxyacetophenone. While Hped (R)-specifically hydrogenates 4-hydroxyacetophenone and acetophenone, Ped does so (S)-specifically with acetophenone.

**Docking of 4-Hydroxyacetophenone into the Active Sites of Hped and Ped**

The substrates 4-hydroxyacetophenone and acetophenone have been docked with the program GLIDE into the active sites of holo-Hped and holo-Ped in order to get an unbiased view of the stereo preference of these two enzymes (fig. 5). The positioning of the keto group was used as a criterion for a catalytically productive pose. In Hped, acetophenone was placed with the highest docking score with the keto carbon with a distance of 3.63 Å to C4 of the nicotinamide and the oxygen forming a hydrogen bond with a distance of 2.87 Å to the hydroxyl group of Tyr\(^ {155} \) (fig. 5a). In Ped, both molecules were placed with the highest docking score in identical positions with the keto carbon atom in a distance of 3.83 Å to the C4 of the nicotinamide ring and the oxygen atom in hydrogen bond distance (3.37 Å) to the hydroxyl group of the active site Tyr\(^ {155} \) (fig. 5b). For 4-hydroxyacetophenone, the third highest ranked pose gave the same catalytically productive placement. The two higher ranked poses form a hydrogen bond to the oxygen of the phosphate (NO1) leading to a larger distance to C4 (>4.0 Å). The binding poses will lead to the (S)-alcohol for Ped and (R)-alcohol for Hped, reflecting the steric requirements of the binding pocket due to Tyr\(^ {93} \) in Ped and Phe\(^ {187} \) in Hped.

**Pathway Inherent Enantioselectivity of Hped and Ped**

The opposite enantioselectivities of Hped (R-specific) and Ped (S-specific) from ‘A. aromaticum’ EbN1 agree with the assumed stereospecificities of the preceding enzymes catalyzing the respective initial steps in the two different degradation pathways. While the proposed 4-ethylphenol methylenehydroxylase (PchCF) has not been purified yet from ‘A. aromaticum’ EbN1, the analogous enzyme from *Pseudomonas putida* JD1 was previously demonstrated to form the (R)-isomer of 1-(4-hydroxyphenyl)-ethanol from p-ethylphenol [Reeve et al., 1990]. In contrast, activity and structural analysis of ethylbenzene dehydrogenase from ‘A. aromaticum’ EbN1...
demonstrated formation of the (S)-isomer of 1-phenylethanol [Kloer et al., 2006; Kniemeyer and Heider, 2001a]. The (S)-enantioselectivity of EbA309 determined by enzymatic measurements explains why this dehydrogenase cannot substitute Hped in oxidation of (1R)-(4-hydroxyphenyl)-ethanol, despite the chromosomal colocalization and coexpression of the coding genes (hped and ebA309).

**Materials and Methods**

**Bacterial Strains and Cultivation**

The cultivation of ‘A. aromaticum’ EbN1 and *E. coli* in liquid and on solid medium was performed as described previously [Rabus and Widdel, 1995; Wöhlbrand and Rabus, 2009]. Strains used and generated in this study are given in table 1.

**Molecular Genetics (Unmarked In-Frame Deletion)**

The isolation of genomic DNA and plasmids was performed according to standard methods [Sambrook and Russel, 2001]. Oligonucleotide primers were designed using the software Lasergene (version 7.0; DNASTAR, Madison, Wis. USA) and purchased from Biomers.net GmbH (Ulm, Germany). Details on primers used in this study are summarized in table 2.

For reasons of unambiguous nomenclature, the new gene designation *hped* is used throughout this report instead of the original gene name *chnA*. The vector for unmarked knockout of *hped* and *ebA309* (pK19 Ω*ebA310_ebA303/tioL*) was based on the suicide plasmid pK19mobsacB [Schäfer et al., 1994] containing 2.6 kbp of the 5’- and 1.6 kbp of the 3’-flanking regions of *hped* and *ebA309*, respectively, to allow for high double-crossover efficiency. The start codon of *ebA309* and the stop codon of *hped* separated by a PstI restriction site were preserved in the knockout construct to

![Fig. 5. Stereo image of NADH and aromatic substrates modeled in active sites of R- and S-selective dehydrogenases from ‘A. aromaticum’ EbN1. a Hped; for visual clarity, Leu^{192} (grey, thin-lined) was not included in the calculation of the protein surface. b Ped, the aromatic substrates 4-hydroxyacetophenone (purple), and acetophenone (salmon) are displayed as overlay (color in online version only). Amino acid side chains which restrict the substrate binding pocket and constitute stereospecificity are Phe^{187} in Hped and Tyr^{93} in Ped (space filling representation, red).](image)
generate an in-frame deletion mutant and avoid polar effects. The 5′- and 3′-regions were amplified by PCR from the genomic DNA of strain EbN1 using a high-fidelity polymerase (Phusion®; Thermo Fisher Scientific, Dreieich, Germany) based on the genome sequence of 'A. aromaticum' EbN1 (version 2.3; Matrix Science Ltd., London, UK) based on the er (version 2.3; Matrix Science Ltd., London, UK) based on the

Preparation of total RNA from substrate-adapted cells of strain EbN1 and subsequent amplification of transcripts by RT-PCR was performed as described [Kühner et al., 2005].

**Proteomics**

Cultures (400 ml each) of 4-hydroxyacetophenone-adapted cells of mutant strains as well as wild type 'A. aromaticum' EbN1 were harvested as described [Champion et al., 1999] to obtain cell material for whole-cell shotgun proteome analysis. Cell disruption, reduction, alkylation and tryptic digest were performed as described recently [Zech et al., 2013]. Tryptic peptides were analyzed using a nanoRSLC system (Thermo Fisher Scientific, Bremen, Germany) in a trap column mode and equipped with an ion-trap mass spectrometer (amazon ETD; Bruker Daltonik GmbH, Bremen, Germany). Protein identification was performed using the ProteinScape platform (version 3.1; Bruker Daltonik GmbH) on an in-house Mascot server (version 2.3; Matrix Science Ltd., London, UK) based on the genome sequence of 'A. aromaticum' EbN1 [Rabus et al., 2005] applying a target-decoy strategy as described by Zech et al. [2013].

**Cloning of hped Gene and Overexpression in E. coli**

The hped gene was amplified by PCR using the oligonucleotides listed in table 2. The reaction mixture contained 130 ng of each oligonucleotide; 10 mM dATP, dCTP, dGTP, and dTTP, respectively; approximately 30 ng of a plasmid from a genomic library of 'A. aromaticum' EbN1 containing hped, 10 μl of 10-times PfuUltra reaction buffer (Agilent Technologies, Santa Clara, Calif., USA), and 1 U of PfuUltra-DNA-Polymerase (Agilent Technologies) in a total volume of 100 μl. The mixture was heated for 5 min at 94°C and the gene was amplified by 30 cycles (annealing: 60 s at 50°C, polymerization: 120 s at 72°C, and melting: 60 s at 94°C) plus a final extension at 72°C for 10 min. PCR products were cleaved with Ndel and BglII, ligated into a pJOE2702 vector

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype and/or characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>'A. aromaticum' EbN1</td>
<td>Wild type</td>
<td>Widdel and Rabus, 2001</td>
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<tr>
<td>EbN1 Δhped/ebA309</td>
<td>Δhped, ΔebA309</td>
<td>This study</td>
</tr>
<tr>
<td>EbN1 hped/ebA309-complemented mutant</td>
<td>Δhped, ΔebA309, pBBR1MCS-4-ΩebA309/hped</td>
<td>This study</td>
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<td>EbN1 hped-complemented mutant</td>
<td>Δhped, ΔebA309, pBBR1MCS-4-Ωhped</td>
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<td>EbN1 ebA309-complemented mutant</td>
<td>Δhped, ΔebA309, pBBR1MCS-4-ΩebA309</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>Pro, thi, hsdR, Tra′, recA, Tra′, Sm′, ΩRP4-TE::Mu-Km::Tn7</td>
<td>Simon et al., 1993</td>
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<td>E. coli TG1 (DSM 6056)</td>
<td>Δ(lac-pro, supE, thi, hsdD5, F traD36, proA′B′, lacI, lacZ ΔM15</td>
<td>Carter et al., 1985</td>
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<table>
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<th>Plasmids</th>
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<tr>
<td>pK19mobbacB</td>
<td>Km&lt;sup&gt;6&lt;/sup&gt;, sacB modified from B. subtilis, lacZα</td>
<td>Schönä et al., 1994</td>
</tr>
<tr>
<td>pK19 ebA310</td>
<td>Km&lt;sup&gt;6&lt;/sup&gt;, sacB modified from B. subtilis, lacZα, ebA310 from strain EbN1</td>
<td>This study</td>
</tr>
<tr>
<td>pK19 ebA310_ ebA303/tioL</td>
<td>Km&lt;sup&gt;6&lt;/sup&gt;, sacB modified from B. subtilis, lacZα, ebA310, ebA303 and part of tioL from strain EbN1</td>
<td>This study</td>
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<tr>
<td>pBBR1MCS-4</td>
<td>Ap&lt;sup&gt;5&lt;/sup&gt;, mob, lacZα</td>
<td>Kovach et al., 1995</td>
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<td>pBBR1MCS-4-Ωhped/ebA309</td>
<td>Ap&lt;sup&gt;5&lt;/sup&gt;, mob, lacZα, hped and ebA309 from strain EbN1</td>
<td>This study</td>
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<tr>
<td>pBBR1MCS-4-Ωhped</td>
<td>Ap&lt;sup&gt;5&lt;/sup&gt;, mob, lacZα, hped from strain EbN1</td>
<td>This study</td>
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<tr>
<td>pBBR1MCS-4-ΩebA309</td>
<td>Ap&lt;sup&gt;5&lt;/sup&gt;, mob, lacZα, ebA309 from strain EbN1</td>
<td>This study</td>
</tr>
<tr>
<td>pJOE2702</td>
<td>ColE1, bla, Ap&lt;sup&gt;5&lt;/sup&gt;, P&lt;sub&gt;PuaBAD&lt;/sub&gt;-rrnB, expression vector</td>
<td>Voll et al., 1996</td>
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<tr>
<td>pJOEdhped</td>
<td>ColE1, bla, Ap&lt;sup&gt;5&lt;/sup&gt;, P&lt;sub&gt;PuaBAD&lt;/sub&gt;-rrnB, hped, expression vector</td>
<td>This study</td>
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</tbody>
</table>

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Purification of the Overproduced Hped from E. coli

Hped was purified essentially according to the following procedure; for further details see section Purification of Hped in the supplemental material.

**Enzyme Assays**

The oxidation of NADH and concomitant carbonyl reduction was measured by incubating 10 μmol of the respective carbonyl compound – added from a 1-M stock solution in DMSO – with 980 μl of potassium phosphate buffer (50 mM, pH 6.5, 0.2 mM NADH, 1 mM MgCl₂). Addition of 10 μl of crude extract (approx. 75 μg of protein) initiated the reaction which was monitored at 340 nm.

Table 2. Oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene</th>
<th>Nucleotide sequence (5′→3′)</th>
<th>Product length (bp)</th>
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<tr>
<td>Gene-specific primer pairs&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>hped_153_F</td>
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<td>TGATCGAAGGGCAAGGGCCGAAAAG GCGGCGGTGTAGGGCCGTAGT</td>
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<td>hped_473_R</td>
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<td>ebA309_311_F</td>
<td>ebA309</td>
<td>AACTCGCGCATCAACAACACTCC</td>
<td>320</td>
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<td>ebA309_630_R</td>
<td>ebA309</td>
<td>ATTCGCCAGACTTCGGCAATTTTC</td>
<td>366</td>
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<tr>
<td>ebA309_931_F</td>
<td>ebA309</td>
<td>ATTCGCCAGACTTCGGCAATTTTC</td>
<td>366</td>
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<tr>
<td>tiol_448_R</td>
<td>tiol</td>
<td>CCCCCGCTGTCGGAGATGGAAGAT C</td>
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<td>tiol_809_F</td>
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<td>ebA309 deletion construct</td>
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<tr>
<td>ebA303_PstI_R</td>
<td>ebA303 and tiol</td>
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<td>ebA310</td>
<td>AACTGCAGCATGAAATAACACCTCGGTTACCGGGTT</td>
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<td>Identification of Δhped/ebA309</td>
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<tr>
<td>tiol_69_R</td>
<td>tiol</td>
<td>GGGCTCATGAGCGCGCGGG</td>
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<tr>
<td>ebA310_1315_F</td>
<td>ebA310</td>
<td>CCGGAAGAAGCGAGCCCCCAGCT</td>
<td>1,945&lt;sup&gt;c&lt;/sup&gt;/361&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Complementation constructs of hped and ebA309</td>
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<td>KOM_ebA309_ApaI_F</td>
<td>ebA309</td>
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<td>KOM_hped_ApaI_F</td>
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<td>KOM_hped_XbaI_R</td>
<td>hped</td>
<td>AAATTCTAGATCTGTCTTCTTGGCATGGCCGATG</td>
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<tr>
<td>Overexpression of hped</td>
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<tr>
<td>hped_fw</td>
<td>hped</td>
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<td></td>
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<tr>
<td>hped_rev</td>
<td>hped</td>
<td>CTGATAGATCTTAGTGAGCGATGAGGATCA</td>
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</tbody>
</table>

<sup>a</sup> R = Reverse primer; F = forward primer. <sup>b</sup> Recognition sites for restriction enzymes are marked in bold type. <sup>c</sup> Wild type strain EbN1. <sup>d</sup> Δhped-ebA309 mutant of strain EbN1.

[Vollf et al., 1996] cleaved with NdeI and BamHI (yielding the expression vector pJOE Hped), and transformed into E. coli strain TG1 [Carter et al., 1985]. Recombinant E. coli cells were grown in LB medium [Sambrook and Russel, 2001] and induced by the addition of 0.2% (w/v) rhamnose. After 10 h of induction, Hped was highly overproduced in soluble form and purified as described below.

**Purification of the Overproduced Hped from E. coli**

Hped was purified essentially according to the following procedure; for further details see section Purification of Hped in the supplemental material. E. coli cells containing the overproduced Hped were resuspended in protease inhibitor-containing Tris/HCl buffer (50 mM, pH 7.5), glass sinter filtered and homogenized with a microfluidizer. The obtained homogenate was stored at -20°C. For initial separation by hydrophobic chromatography, the homogenate was amended with ammonium sulfate (20% saturation) and applied to a phenyl-sepharose FF column (Pharmacia, Upsala, Sweden). The bulk of enzymatic activity was eluted with a linear gradient to 20 mM of Tris/HCl (pH 7.5) with 10% isopropanol.
lowing temperature program: start at 90 °C, increase temperature by 3 °C min−1 to 140 °C and hold 5 min at 140°C.

Enantioselectivity

The stereoselectivity of EbA309 and Hped was determined in the reductive mode in batch reactions with an excess of a secondary alcohol as a hydrogen source. Therewith, the production of chiral compounds from prochiral starting material is monitored rather than the depletion of one of the two enantiomers, which is the case in chiral resolution of a racemic alcohol. This approach (reductive reaction direction) is considered to yield a lower experimental error. 150 μg of crude protein extract containing EbA309 and Hped, respectively, was mixed with 50 μmol of acetophenone and 50 μmol of NAD⁺ in potassium phosphate buffer (50 mM, pH 6.5; 1 mM MgCl₂) and 1.3 mmol of propan-2-ol. The resulting product mixture was extracted with one volume of riBME and analyzed by chiral GC equipped with a Hydrodex b-6TBDM column (25 m; Mache

Carmona M, Zamarro MT, Blázquez B, Durante-rrey-Nagel, Düren, Germany). The GC was operated with the following temperature program: start at 90°C, increase temperature by 3°C min−1 to 140°C and hold 5 min at 140°C.

Protein Crystallization and Data Collection

Crystals of purified Hped were grown with the sitting drop method in a buffer containing 30% PEG 6000, 0.1 M Na-cacodylate (pH 6.5) and 0.3 M Mg-acetate. Prior to data collection, one crystal was soaked in 1 mM NAD⁺ for 4 h. The data for the apo- and for the NAD⁺-soaked form were collected at the PX III beam line at the synchrotron Swiss Light Source (Villigen, Switzerland). The crystals were flash frozen in a cold nitrogen stream at 100 K. The crystals belong to the monoclinic space group C2 with cell parameters of a = 121.5 Å, b = 55.1 Å, c = 86.3 Å and β = 134.4°. An overview of the X-ray data collection is provided in online supplementary table 1.

Structure Determination and Refinement

The structure of the holo-form of Hped was solved by molecular replacement with the program MOLREP as part of the CCP4 package [Winn et al., 2011]. The phasing model was levodione re-
ductase (PDB accession code 1IY8) [Sogabe et al., 2003], which has 38% amino acid sequence identity with Hped. Monomer A was pruned with the program CHAINSAW from the CCP4 package. The Matthews coefficient anticipated two monomers per asym-
metr unit. MOLREP produced a solution with a dimer for the NAD⁺ data set. Subsequent cycles of refinement with the program refmac5 from the CCP4 suite and model building with COOT [Emsley et al., 2010] further decreased the R-factor. Water molecules, 5 Mg²⁺ ions, 5 acetate molecules and one partially occupied sulfobetaine molecule (occupancy 0.5 for the propane sulfonate chain) were added as suggested by the electron density and the protein environment. The refinement procedure resulted in a final model with an R-factor of 0.11 (Rfree = 0.14) and good stereochem-
istry. The final protein model of the holo-enzyme was used as the starting model for the apo-structure. Several cycles of refinement und manual rebuilding resulted in an R-factor of 0.17 (Rfree = 0.22). An overview of the structure refinement statistics is given in table S1 in the supplementary material. Ramachandran plots were generated using the program COOT [Emsley et al., 2010].

Docking

The substrates 4-hydroxyacetophenone and acetophenone were docked with the docking software GLIDE (Schrödinger Inc., Portland, Oreg., USA) [Halgren et al., 2004] into the active sites of Hped and Ped. The molecules were created with the 2D sketcher and converted to a 3D structure, and the geometrics were optim-
ized. The polar hydrogens of the active site residues Tyr135 and Ser136 were placed manually in reasonable positions. Default param-
eters were used for the preparation of the ligands with LigPrep, the grid generation and GLIDE SP docking.

Database Submission

Coordinates of the apo- and holo-structures have been depos-
ited in the Protein Data Bank (entries 4ure and 4urf).

Acknowledgements

We thank R. Reinhardt (Köln) for providing cloned hped, Petra Reis (Ludwigshafen) for crystallization and Wolfgang Houy (Ludwigshafen) for data collection. This work was supported by the FOL program of the Carl von Ossietzky University Oldenburg.

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new derivatives of the broad-host-range clon-
ning vector pBR1MCS carrying different an-

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