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# Identification and molecular characterization of the eugenol hydroxylase genes *(ehyA/ehyB)* of *Pseudomonas* sp. strain HR199

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**Abstract** The gene loci *ehyA* and *ehyB*, which are involved in the bioconversion of eugenol to coniferyl alcohol by *Pseudomonas* sp. strain HR199 (DSM 7063), were identified as the structural genes of a eugenol hydroxylase that represents an enzyme of the flavocytochrome *c* class. These genes were localized downstream of the eugenol catabolism genes *vanA* and *vanB*, encoding vanillate-*O*-demethylase, on an *Eco*RI fragment (E230) that has recently been cloned from a *Pseudomonas* sp. strain HR199 genomic library. The gene encoding the cytochrome *c* subunit *(ehyA)* was identified on a subfragment (K18) of E230 by complementation of a nitrosoguanidine-induced, eugenol-negative mutant of strain HR199. The nucleotide sequences of fragment K18 and adjacent regions were determined, revealing open reading frames of 354 and 1,554 bp that represent *ehyA* and *ehyB*, respectively. These genes are most probably organized in one operon together with a third open reading frame (ORF2) of 687 bp that was located between *ehyA* and *ehyB*. The deduced amino acid sequences of *ehyA* and *ehyB* exhibited up to 29 and 55% amino acid identity to the corresponding subunits of *p*-cresol methylhydroxylase from *Pseudomonas putida*. Moreover, the amino-terminal sequences of the α- and β-subunits reported recently for a eugenol dehydrogenase of *Pseudomonas fluorescens* E118 corresponded well with appropriate regions of *ehyA* and *ehyB*. The sequence of ORF2 and the deduced amino acid sequence exhibited no significant similarities to any DNA or amino acid sequence from the databases. The eugenol hydroxylase genes were amplified by PCR, cloned in pBluescript SK$^-$, and functionally expressed in *Escherichia coli*. Transfer of a DNA fragment comprising *ehyA* and *ehyB* to various strains of *Pseudomonas* species that were unable to utilize eugenol as a carbon source conferred to these bacteria the ability to grow on this substrate.

**Key words** *Pseudomonas* · Eugenol hydroxylase · Flavocytochrome *c* · Formaldehyde dehydrogenase · γ-Glutamylcysteine synthetase · Vanillin production · *ehyA* · *ehyB* · *fdh* · *gcs*

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## Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the characteristic aroma component of the vanilla pod and is frequently used for the production of flavors for foods and of fragrances for perfumes. At present, artificial or nature-identical vanillin is produced mostly from petrochemicals and from lignin (Clark 1990). Since vanillin occurs as an intermediate in the catabolism of phenolic stilbenes, eugenol (4-allyl-2-methoxyphenol), ferulate (4-hydroxy-3-methoxycinnamate), and lignin (Toms and Wood 1970; Chen et al. 1982; Tadasa and Kayanara 1983; Shiotsu et al. 1989), it might be also produced by biotransformation of these substrates (Hagedorn and Kaphammer 1994; Rabenhorst 1996).

We are currently investigating a process based on the biotransformation of eugenol, which is the main component of the essential oil of the clove tree *Syzygium aromaticum*, by *Pseudomonas* sp. strain HR199 (Steinbüchel et al. 1998). Eugenol is catabolized by this organism via coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol), coniferyl aldehyde (4-hydroxy-3-methoxycinnamyl aldehyde), ferulate, vanillin, vanillate (4-hydroxy-3-methoxybenzoate), and protocatechuate (3,4-dihydroxybenzoate) (Rabenhorst 1996; Overhage et al. 1999b; Fig. 1), which is further metabolized by *ortho*-cleavage (Overhage et al. 1999). The first study on the bacterial degradation of eugenol was initiated by Tadasa (1977) on a *Corynebacterium* sp.; Tadasa also proposed the formation of an epoxide (eugenol oxide) as the initial reaction of the eugenol catabolism in a *Pseudomonas* sp. (Tadasa and Kayanara 1983). It has been shown that a *p*-quinone me-

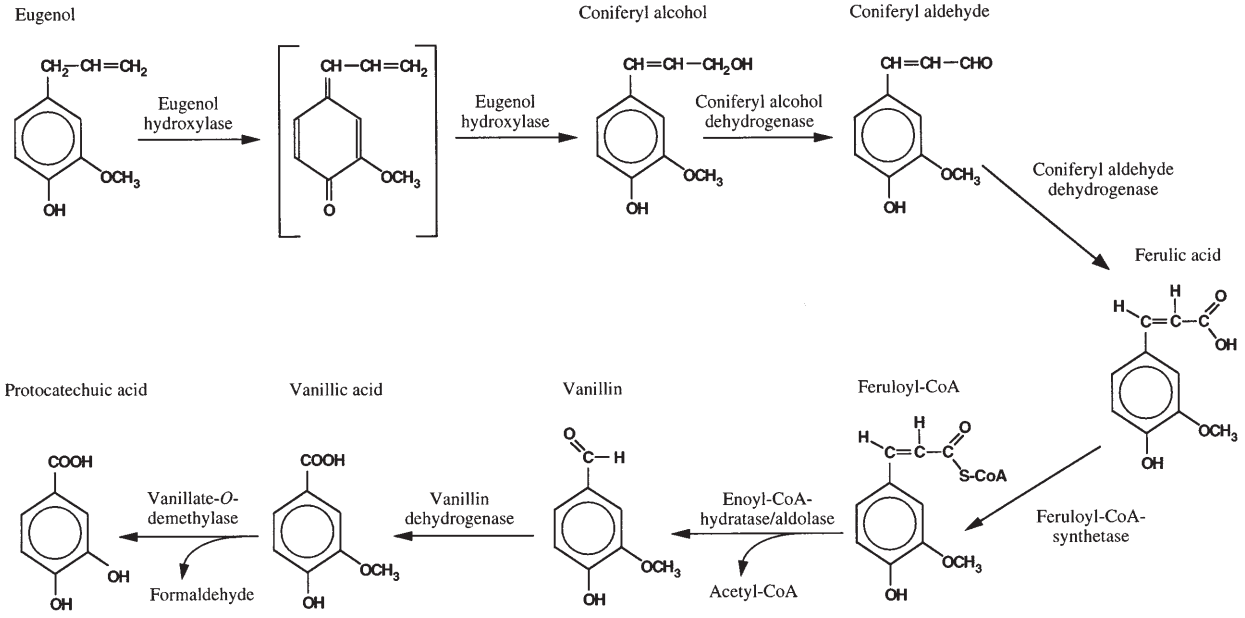


**Fig. 1** Proposed route for the catabolism of eugenol in *Pseudomonas* sp. strain HR199

thide is an intermediate in the conversion of eugenol to coniferyl alcohol catalyzed by vanillyl alcohol oxidase of *Penicillium simplicissimum* (Fraaije et al. 1995; Fraaije and Van Berkel 1997). Recently, new interest has been focused on eugenol degradation with respect to the development of a biotransformation process to produce natural vanillin (Rabenhorst and Hopp 1990). A new *Pseudomonas* sp. (strain HR199) that is able to produce methoxyphenol-type aroma chemicals by biotransformation of eugenol has been isolated (Rabenhorst 1996). To reveal the physiological and genetic basis for this biotransformation, studies were initiated to identify the genes that are essentially involved in the degradation of eugenol.

Genes encoding a eugenol hydroxylase have never been cloned. In the present study, we describe the molecular characterization and heterologous expression of the eugenol hydroxylase genes *ehyA* and *ehyB* of *Pseudomonas* sp. strain HR199. Our data confirm the suggestion that eugenol hydroxylase belongs to the family of flavocytochromes *c*, as reported recently for a eugenol dehydrogenase of *Pseudomonas fluorescens* E118 (Furukawa et al. 1998).

## Materials and methods

### Bacterial strains and culture conditions

The strains of *Pseudomonas* sp. and *Escherichia coli* and the plasmids used in this study are listed in Table 1. Cells of *E. coli* were grown at 37 °C in Luria-Bertani (LB) or in M9 minimal medium (Sambrook et al. 1989). Cells of *Pseudomonas* sp. strains were grown at 30 °C either in a nutrient broth (NB) medium (0.8%, w/v; Bacto, Difco), or in MM (Schlegel et al. 1961) or HR-MM (Rabenhorst 1996) mineral salts media supplemented with carbon sources as indicated in the text. Ferulic acid, vanillin, vanillic acid, and protocatechuic acid were dissolved in dimethyl sulfoxide and were added to the medium at final concentrations of 0.1% (w/v). Eugenol was added directly to the medium at a final concentration of 0.1% (v/v). Tetracycline and kanamycin were used at final concentrations of 25 and 300 μg/ml, respectively for *Pseudomonas* sp. Growth of the bacteria was monitored with a Klett-Summerson photometer. Samples were taken from the cultures, and cells were removed by centrifugation. The obtained culture supernatants were analyzed by HPLC with respect to the appearance or disappearance of catabolic intermediates as described below.

### Analytical methods

Using a Knauer HPLC apparatus, culture supernatants were analyzed for excreted intermediates of the eugenol catabolism by liquid chromatography without prior extraction. Intermediates were separated by reversed-phase chromatography on Nucleosil-100 C-18 (particle size, 5 μm; column, 250 × 4.0 mm) with a gradient of 0.1% (v/v) formic acid (eluant A) and acetonitrile (eluant B) in a range of 20– 100% (v/v) eluant B and at a flow rate of 1 ml/min. For quantification, all intermediates were calibrated with external standards. The compounds were identified by their retention times, and the corresponding spectra, which were identified with a diode array detector (WellChrom Diodenarray-Detektor K-2150; Knauer, Berlin, Germany). Proteins were separated under nondenaturating conditions in 7.4% (w/v) polyacrylamide gels as described by Stegemann et al. (1973), and under denaturating conditions in 11.5% (w/v) polyacrylamide gels according to Laemmli (1970). The proteins in the gels were stained with Serva Blue R. To stain gels for eugenol hydroxylase activity, the gels were incubated at 30 °C in the dark in 100 mM potassium phosphate buffer (pH 7.0) containing 0.04% (w/v) *p*-nitroblue tetrazolium chloride, 0.003% (w/v) phenazine methosulfate, and 1 mM eugenol.

### Enzyme assay

Cells were disrupted either by a twofold French press passage at 96 MPa or by sonication (1 min/ml of cell suspension with an amplitude of 40 μm) with a Bandelin Sonopuls GM200 ultrasonic disintegrator. The soluble fractions of crude extracts were obtained by centrifugation at 100,000 × *g* at 4 °C for 1 h. The eugenol hydroxylase activity was assayed photometrically at 30 °C in a total volume of 1 ml containing 5 μmol eugenol, 0.67 μmol phenazine methosulfate, 0.1 μmol 2,6-dichlorophenol-indophenol (DCPIP),

**Table 1** Bacterial strains and plasmids used in this study (*DSMZ* Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; *LMD* Laboratorium voor Microbiologie, Technische Universiteit, Delft, The Netherlands)

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *Pseudomonas* sp. | | |
| HR199 | Wild-type, eugenol-positive, ferulic-acid-positive | DSMZ 7063; Rabenhorst (1996) |
| SK6165[a] | Eugenol-negative, ferulic-acid-positive | This study |
| *Pseudomonas* sp. | | |
| D1A | Eugenol-negative, ferulic-acid-positive | A. Viale |
| D1B | Eugenol-negative, ferulic-acid-positive | A. Viale |
| DSMZ 1455 | Eugenol-negative, ferulic-acid-positive | DSMZ 1455 |
| DSMZ 18 | Eugenol-negative, ferulic-acid-positive | DSMZ 18 |
| DSMZ 51 | Eugenol-negative, ferulic-acid-positive | DSMZ 51 |
| *Pseudomonas fluorescens* type B | Eugenol-negative, ferulic-acid-negative | DSMZ 50108 |
| *Pseudomonas stutzeri* | Eugenol-negative, ferulic-acid-negative | DSMZ 50027 |
| *Pseudomonas fragi* | Eugenol-negative, ferulic-acid-negative | DSMZ 3456 |
| *Pseudomonas testosteroni* | Eugenol-negative, ferulic-acid-negative | LMD 3324 |
| *Escherichia coli* | | |
| XL1-Blue | *recA1 endA1 gyrA96 thi hsdR17* ($r_K^- m_K^+$) *supE44relA1*, $\lambda^-$, *lac* [F′ *proAB lacI*$^q$ZΔM15, Tn*10*(Tet)] | Bullock et al. (1987) |
| S17-1 | *recA*; harboring the *tra* genes of plasmid RP4 in the chromosome, *proA, thi-1* | Simon et al. (1983) |
| Plasmids | | |
| pVK100 | Tc$^r$, Km$^r$, cosmid, broad host range | Knauf and Nester (1982) |
| pMP92 | Tc$^r$, broad host range | Spaink et al. (1987) |
| pBluescript SK$^-$ | Ap$^r$ *lacPOZ′*, $T_7$ and $T_3$ promoter | Stratagene (San Diego, Calif., USA) |
| pE207 | pVK100 harboring fragment E230 | Priefert et al. (1997) |
| pSKE230 | pBluescript SK$^-$ harboring fragment E230 | Priefert et al. (1997) |

[a] SK6165 is a mutant of *Pseudomonas* sp. strain HR199

and an appropriate amount of enzyme in 100 mM potassium phosphate buffer (pH 7.0). The reaction was followed by measuring the initial absorbance changes at 600 nm due to the reduction of DCPIP ($\varepsilon$ = 21 cm$^2$ μmol$^{-1}$). The activities determined with the spectrophotometric test were confirmed by measuring the eugenol and coniferyl alcohol concentrations by HPLC analysis of corresponding stopped reaction tests. The amount of soluble protein present was determined as described by Lowry et al. (1951).

## Isolation, manipulation, analysis, and transfer of DNA

Plasmid DNA, DNA restriction fragments, and PCR products were isolated and analyzed by standard methods according to references compiled in a previous study (Priefert et al. 1991). Competent cells of *E. coli* were prepared and transformed by the $CaCl_2$ procedure (Hanahan 1983). Conjugations of *E. coli* S17-1 (donor) harboring hybrid plasmids and of *Pseudomonas* sp. (recipient) were performed on solidified NB medium (Friedrich et al. 1981) or by a "minicomplementation method" as described previously (Priefert et al. 1997). For the construction of a genomic library of *Pseudomonas* sp. strain HR199, genomic DNA partially digested with *Eco*RI was ligated with *Eco*RI-linearized cosmid pVK100. The ligation mixtures were packaged in λ-particles and were subsequently transduced into *E. coli* S17-1. Transductants (1,330) were selected on LB-Tc agar plates, and the hybrid cosmids of these strains were transferred to the eugenol-negative mutant SK6165 by conjugation. For DNA sequence determination, the dideoxy chain-termination method (Sanger et al. 1977) was applied. The nucleotide sequences were determined with a 4000L DNA sequencer (LI-COR, Biotechnology Division, Lincoln, Neb., USA). A Thermo Sequenase fluorescent-labeled primer cycle-sequencing kit with 7-deaza-dGTP (Amersham, Little Chalfont, Buckinghamshire, UK) was used as specified by the manufacturer together with synthetic fluorescent-labeled oligonucleotides as primers. The primer-hopping strategy was employed (Strauss et al. 1986). Nucleotide and amino acid sequences were analyzed with the Genetics Computer Group sequence analysis software package (GCG Package, version 6.2, June 1990) as described by Devereux et al. (1984).

## Materials

Nutrient broth (NB) was from Oxoid (Basingstoke, Great Britain). Restriction endonucleases, T4 DNA ligase, λ-DNA, and enzymes and substrates used in the enzyme assays were obtained from Boehringer (Mannheim, Germany) or from Gibco/BRL (Eggenstein, Germany). Agarose type NA was purchased from Pharmacia-LKB (Uppsala, Sweden). Synthetic oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). All other chemicals were from Haarmann & Reimer (Holzminden, Germany), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), Serva (Heidelberg, Germany), or Sigma (Deisenhofen, Germany).

## Nucleotide sequence accession number

The nucleotide and amino acid sequence data reported in this paper were submitted to the EMBL, GenBank, and DDBJ nucleotide sequence databases and are listed under accession no. AJ243941.

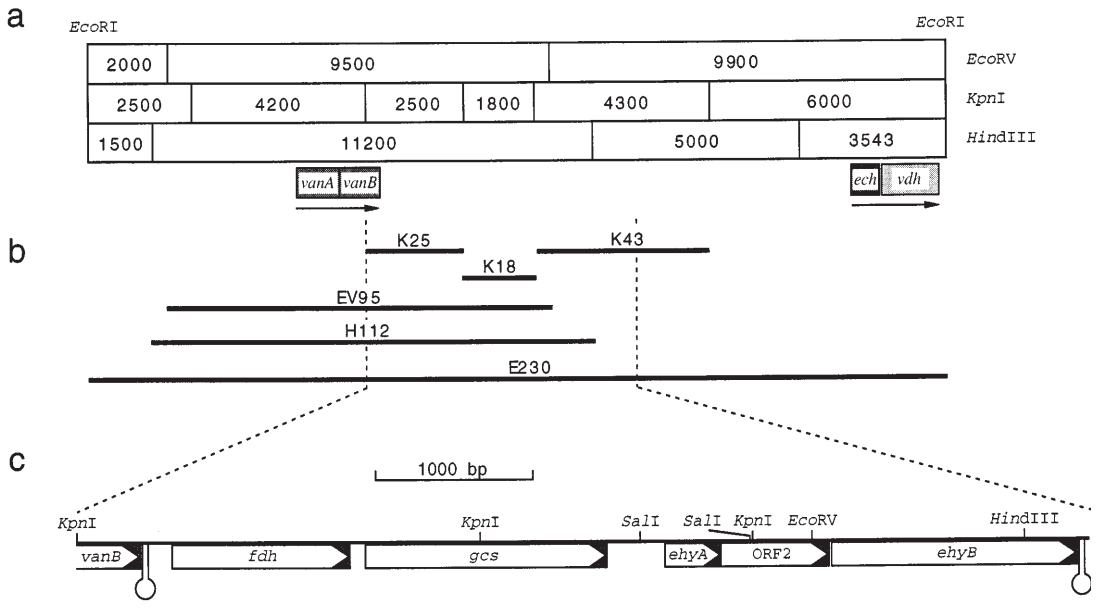


**Fig. 2 a–c** Localization of *ehyA*, *ehyB*, ORF2, *fdh*, *gcs*, *vanA*, *vanB*, *ech*, and *vdh*. **a** Physical map of fragment E230; **b** relevant subfragments; and **c** structural genes of the eugenol hydroxylase (*ehyA* and *ehyB*), formaldehyde dehydrogenase *(fdh)*, and γ-glutamyl-cysteine synthetases *(gcs)*

## Results

### Cloning of the genes involved in the eugenol degradation pathway

*Pseudomonas* sp. strain HR199 is able to utilize eugenol as sole carbon source for growth. For identification of the genes that are involved in the first steps of the degradation pathway, mutants that were unable to grow on eugenol but that retained the ability to grow on ferulic acid were isolated after nitrosoguanidine mutagenesis. One of these mutants (SK6165) was complemented by the hybrid cosmid pE207 harboring a 23-kb *Eco*RI fragment (E230), which had been identified in a genomic library of *Pseudomonas* sp. strain HR199 (Priefert et al. 1997).

### Subcloning of the genes *ehyA*, *ehyB*, and *fdh*

Fragment E230 has recently been cloned in pBluescript SK⁻, resulting in the hybrid plasmid pSKE230, and a physical map of E230 has been obtained (Priefert et al. 1997). This fragment harbors the vanillin catabolism genes *vanA*, *vanB*, and *vdh* encoding the subunits of vanillate-*O*-demethylase and the vanillin dehydrogenase, respectively (Priefert et al. 1997). The open reading frame *(ech)* identified upstream of *vdh* (Fig. 2) encodes an enoyl-CoA hydratase/aldolase that has been shown to be involved in eugenol and ferulic acid catabolism (Gasson et al. 1998; Overhage et al. 1999b).

Fragment E230 was isolated from *Eco*RI-digested plasmid pSKE230 and was digested with *Kpn*I. The resulting 4.2- (K42), 2.5- (K25), 1.8- (K18), and 4.3-kb (K43) *Kpn*I fragments and also the 2.5- (EK25) and 6.0-kb (KE60) *Kpn*I-*Eco*RI fragments were cloned in pMP92 (Fig. 2). After conjugative transfer of the resulting plasmids from corresponding *E. coli* S17-1 strains to the eugenol-negative mutant SK6165, complementation was achieved only with fragment K18. Fragments K18, K25, and K43 were cloned in pBluescript SK⁻, and the resulting hybrid plasmids pSKK18, pSKK25, and pSKK43 were used as template DNA in sequencing reactions.

### Nucleotide sequence of fragments K25, K18, and K43

The nucleotide sequences of fragments K25 and K18 and a part of fragment K43 were determined (AJ243941). The coherence of these fragments was confirmed by sequencing the overlapping fragments EV95 and H112 (Fig. 2) in the regions of the *Kpn*I sites. An open reading frame of 354 bp (ORF1) whose putative translational product exhibited significant similarity with cytochrome *c* subunits of flavocytochromes *c* from various other bacteria (Fig. 3) and which was referred to as *ehyA* was identified on fragment K18. Thus, mutant SK6165 most probably lacked a functional cytochrome *c* subunit (α-subunit) of the eugenol hydroxylase. The translational stop codon of *ehyA* at position 4183 (AJ243941) overlapped with the ATG start codon of a second open reading frame of 687 bp (ORF2). The ATG start codon of a third open reading frame of 1,554 bp (ORF3) was identified 15 bp downstream of the translational stop codon of ORF2 at position 4865 (AJ243941); it was referred to as *ehyB* because its putative translational product exhibited significant similarity with β-subunits of flavocytochromes *c* from various other bacteria (Fig. 4). Putative Shine-Dalgarno sequences (AGG, AGG, and GGAG) preceded the ATG start codons of *ehyA*, *ehyB*,

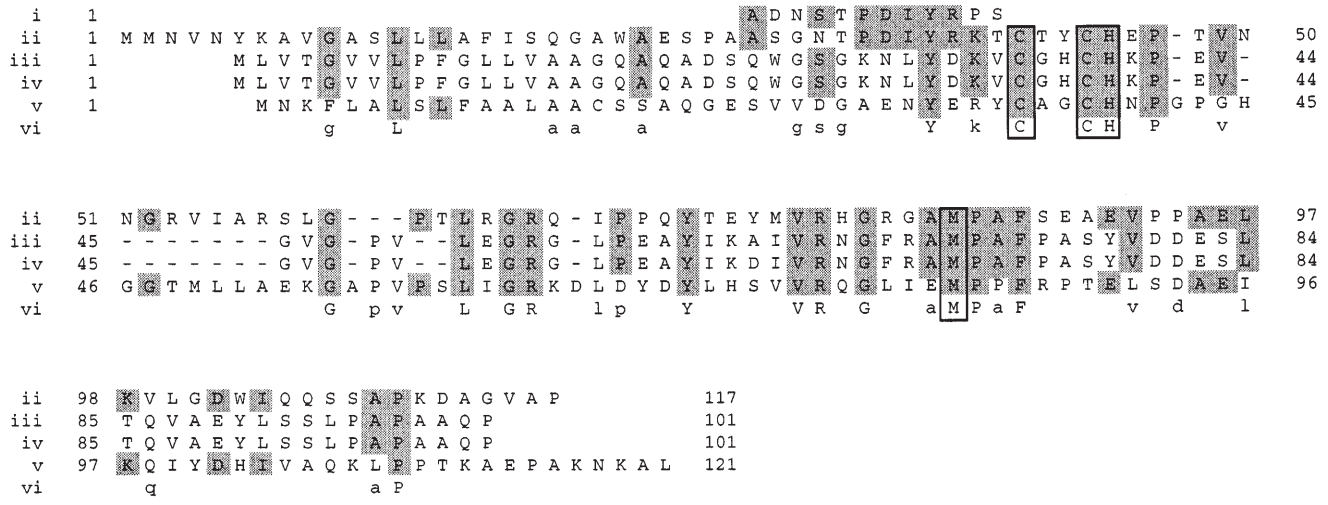

**Fig. 3** Alignment of the α-subunit of eugenol hydroxylase from *Pseudomonas* sp. strain HR199 with cytochrome *c* subunits of flavocytochromes *c* from various sources. The amino acid sequences of the α-subunit of eugenol dehydrogenase (N-terminus) from *Pseudomonas fluorescens* E118 (Furukawa et al. 1998; *lane i*), of the cytochrome *c* subunit of *p*-cresol methylhydroxylase (PchC) from *Pseudomonas putida* NCIMB 9866 (Kim et al. 1994; *lane iii*), of the cytochrome *c* subunit of *p*-cresol methylhydroxylase (PchC) from *Pseudomonas putida* NCIMB 9869 (Kim et al. 1994; *lane iv*), and of the cytochrome subunit of a *p*-cresol methylhydroxylase-related flavocytochrome (plasmid pNL1 encoded; PchC) from *Sphingomonas aromaticivorans* F199 (Romine et al. 1999; *lane v*) were aligned to the sequence of the cytochrome *c* subunit of eugenol hydroxylase from *Pseudomonas* sp. strain HR199 deduced from *ehyA (lane ii)*. Amino acids are specified by standard *one-letter abbreviations*. *Gaps* (–) were introduced into the sequences in order to improve the alignment. Amino acid residues that are identical with the *Pseudomonas* sp. HR199 cytochrome *c* subunit of eugenol hydroxylase at one particular sequence position are *shaded*. The consensus sequence is given in *lane vi*. Amino acid residues, which according to Mathews et al. (1991) are involved in binding of the heme group, are indicated by *boxes*

and ORF2 at distances of 8, 8, and 7 nucleotides, respectively. An inverted repeat that may represent a factor-independent transcriptional terminator was identified 10 bp downstream from the translational stop codon of *ehyB* (AJ243941). The relatively high G+C content of the sequenced genes resulted in a bias for G and C in the third ("wobble") position of each codon. The G+C contents for the different codon positions of *ehyA*, *ehyB*, and ORF2 corresponded well with the theoretical values calculated according to the method of Bibb et al. (1984). In addition, the codon usages of *ehyA*, *ehyB*, and ORF2 were very similar to the codon usage for the genes *vdh*, *vanA*, *vanB*, *calB*, *pcaG*, and *pcaH* of this bacterium (Priefert et al. 1997; Achterholt et al. 1998; Overhage et al. 1999), indicating that *ehyA*, *ehyB*, and ORF2 represent coding regions of *Pseudomonas* sp. strain HR199.

Upstream of *ehyA*, ORF4 and ORF5 were identified; their putative translational products exhibited significant similarities with glutathione-dependent formaldehyde dehydrogenases and γ-glutamylcysteine synthetases, and they were therefore referred to as *fdh* and *gcs*, respectively. The translational stop codon of *vanB* has been identified 200 bp upstream of *fdh* and has been characterized recently (Priefert et al. 1997).

## Deduced properties and putative functions of the *ehyA* and *ehyB* gene products

The relative molecular masses of the α- and β-subunits of eugenol hydroxylase, calculated from the amino acid sequence deduced from genes *ehyA* and *ehyB*, were 12,594 and 57,261 Da, respectively. Since the amino acid sequence deduced from gene *ehyA* exhibited a typical leader peptide structure (amino acids 1–28), the calculated mass of the *ehyA* gene product was higher than the molecular masses reported for other α-subunits of flavocytochromes *c*, e.g., 8,780 for the α-subunit of *p*-cresol methylhydroxylase from *Pseudomonas putida* (McIntire et al. 1986) or

**Fig. 4** Alignment of the β-subunit of eugenol hydroxylase from ▶ *Pseudomonas* sp. strain HR199 with flavoprotein subunits of flavocytochromes from various sources and vanillyl-alcohol oxidase from *Penicillium simplicissimum*. The amino acid sequences of the β-subunit of eugenol dehydrogenase (N-terminus) from *Pseudomonas fluorescens* E118 (Furukawa et al. 1998; *lane i*), of the flavoprotein subunit of *p*-cresol methylhydroxylase (PchF) from *Pseudomonas putida* NCIMB 9866 and NCIMB 9869 (Kim et al. 1994; Cronin et al. 1999; *lane iii*), of the flavoprotein subunit of a *p*-cresol methylhydroxylase-related flavocytochrome (plasmid pNL1 encoded; PchFa) from *Sphingomonas aromaticivorans* F199 (Romine et al. 1999; *lane iv*), of the flavoprotein subunit of a *p*-cresol methylhydroxylase-related flavocytochrome (plasmid pNL1 encoded; PchFb) from *Sphingomonas aromaticivorans* F199 (Romine et al. 1999; *lane v*), and of vanillyl-alcohol oxidase (VaoA) from *Penicillium simplicissimum* (Benen et al. 1998; *lane vi*) were aligned to the sequence of the flavoprotein subunit of eugenol hydroxylase from *Pseudomonas* sp. strain HR199 deduced from *ehyB (lane ii)*. Amino acids are specified by standard *one-letter abbreviations*. *Gaps* (–) were introduced into the sequences in order to improve the alignment. Amino acid residues that are identical to the *Pseudomonas* sp. HR199 flavoprotein subunit of eugenol hydroxylase at one particular sequence position are *shaded*. The consensus sequence is given in *lane vii*. The flavin-binding tyrosine residue according to Kim et al. (1994, 1995) is indicated by a *box*

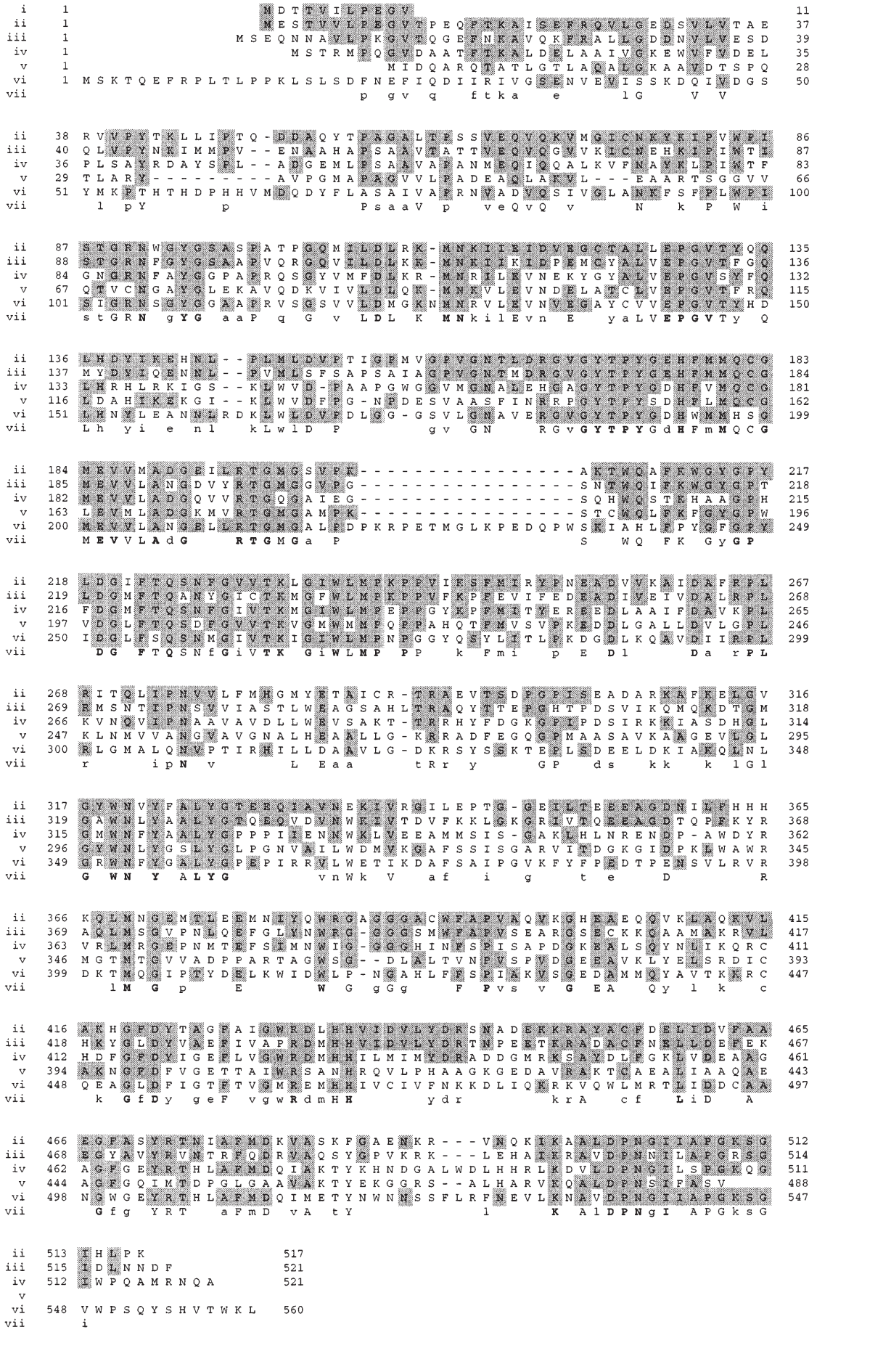

**Table 2** Expression of the genes *ehyA* and *ehyB* of *Pseudomonas* sp. strain HR199 in the eugenol-negative mutant SK6165 and in *Escherichia coli*. Cells of *Pseudomonas* strains were grown at 30 °C in MM containing eugenol as a carbon source to the late exponential phase and harvested. Cells of recombinant strains of *E. coli* were grown for 12 h at 37 °C in LB medium. To induce expression from the *lacZ* promoter, the recombinant strains of *E. coli* harboring pBluescript SK– derivatives were cultivated in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The eugenol hydroxylase activities were determined in soluble extracts at 30 °C by the photometric assay as described in Materials and methods

| Bacterial strain | Plasmid (inducer) | Specific activity of eugenol hydroxylase (U/mg protein) |
|---|---|---|
| *Pseudomonas* sp. strain HR199 | – | 0.041 |
| *Pseudomonas* mutant SK6165 | – | < 0.001 |
| *Pseudomonas* mutant SK6165 | pE207 | 0.022 |
| *Escherichia coli* XL1-Blue | pBluescript SK– (+IPTG) | < 0.001 |
| *Escherichia coli* XL1-Blue | pSK*ehy* (–IPTG) | 0.009 |
| *Escherichia coli* XL1-Blue | pSK*ehy* (+ IPTG) | 0.160 |

10,000 for the cytochrome *c* subunit of eugenol dehydrogenase from *Pseudomonas fluorescens* E118 (Furukawa et al. 1998). The mass calculated for the *ehyB* gene product corresponded well with those reported for other β-subunits of flavocytochromes *c*, e.g., 57,907 for the β-subunit of *p*-cresol methylhydroxylase from *Pseudomonas putida* (Cronin et al. 1999) or 58,000 for the flavoprotein subunit of eugenol dehydrogenase from *Pseudomonas fluorescens* E118 (Furukawa et al. 1998).

The amino acid sequences deduced from genes *ehyA* and *ehyB* were compared with those collected in GeneBank. Highest sequence similarity was obtained with the *p*-cresol methylhydroxylase of *Pseudomonas putida* (29 and 55% identical amino acids for the α- and β-subunits, respectively). The relationships of the α- and β-subunits of the eugenol hydroxylase from *Pseudomonas* sp. strain HR199 to proteins from other sources are shown in Figs. 3 and 4.

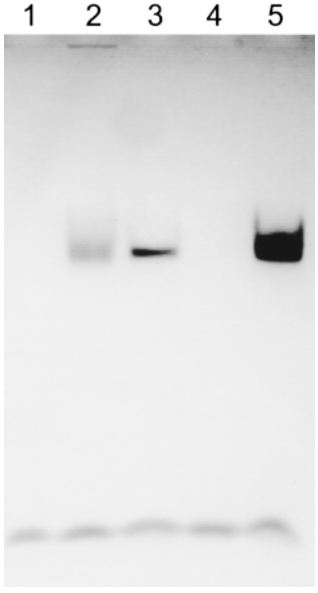


**Fig. 5** Expression of *Pseudomonas* sp. HR199 *ehyAB* genes in mutant *Pseudomonas* sp. strain SK6165 grown in the presence of eugenol and in *E. coli* XL1-Blue. Cytoplasmic fractions obtained from *Pseudomonas* sp. cells grown on gluconate plus eugenol or from *E. coli* cells grown for 12 h in LB in the presence of tetracycline (12.5 μg/ml) and ampicillin (100 μg/ml) were separated in a 7.4% (w/v) polyacrylamide gel as described in Materials and methods, and were stained for eugenol hydroxylase. *Lanes 1 E. coli* XL1-Blue (pBluescript SK–), *2 E. coli* XL1-Blue (pSK*ehy*), *3 Pseudomonas* sp. strain HR199, *4 Pseudomonas* sp. strain SK6165, and *5 Pseudomonas* sp. strain SK6165 (pE207)

## Expression of *ehyA* and *ehyB* from *Pseudomonas* sp. strain HR199 in the eugenol-negative mutant SK6165, and heterologous expression in *E. coli*

Mutant SK6165 was not able to grow on solidified medium with eugenol as sole carbon source and lacked eugenol hydroxylase activity (Table 2; Fig. 5). The hybrid cosmid pE207 was conjugatively transferred from the corresponding *E. coli* S17-1 strain to this mutant. This plasmid restored the ability to grow on eugenol in the corresponding transconjugants. Moreover, these transconjugants exhibited eugenol hydroxylase activities of approximately half the activity obtained with the wild-type (Table 2; Fig. 5).

Genes *ehyA* and *ehyB* were also heterologously expressed in *E. coli*. Genes *ehyA* and *ehyB* were amplified together with ORF2 in a PCR reaction using the primers PCRup (5′-AAAAGAGCTCGGCAGTGACACTTCAAAACAAGAAGGGC-3′), which corresponded to the sequence from position 3,796 to position 3,825 (AJ243941), and PCRdown (5′-AAAAGCGGCCGCCCGCCTCACGAACACGCATTATTTGGG-3′), which was complementary to the sequence from position 6428 to position 6454 (AJ243941). Since the upstream primer exhibited a *Sac*I site and the downstream primer exhibited a *Not*I site, the PCR product was cloned in pBluescript SK– with genes *ehyA* and *ehyB* colinear to and downstream of the *lacZ* promoter. The resulting hybrid plasmid pSK*ehy* conferred eugenol hydroxylase activity to recombinant strains of *E. coli* XL1-Blue (Fig. 5). After growth of this strain in the presence of the inducer IPTG, a eugenol hydroxylase activity of 0.16 U/mg protein was obtained (Table 2).

## Heterologous expression of *ehyA* and *ehyB* from *Pseudomonas* sp. strain HR199 in other *Pseudomonas* strains

The hybrid cosmid pE207 harboring fragment E230 was conjugationally transferred from corresponding *E. coli* S17-1 strains (donor) to the *Pseudomonas* strains (recipients) D1A, D1B, DSMZ 1455, DSMZ 18, DSMZ 51, *Pseudomonas fluorescens* type B, *Pseudomonas stutzeri*, *Pseudomonas fragi*, and *Pseudomonas testosteroni*, which were able neither to grow on eugenol as the sole carbon source nor to convert eugenol to coniferyl alcohol. During growth of the obtained transconjugants on solidified MM

in the presence of eugenol, a bright yellow substance (which was most probably coniferyl aldehyde) was excreted into the agar. To confirm the conversion of eugenol to coniferyl alcohol by these transconjugants, the transconjugants were grown in liquid cultures with 0.5% (w/v) sodium gluconate and 0.1% (v/v) eugenol, and the occurrence of coniferyl alcohol and coniferyl aldehyde in the culture supernatants was proven by HPLC analyses. Transconjugants that only harbored the vector pVK100 as a negative control (and which were treated in the same way) showed no conversion. This indicated the expression of *ehyA* and *ehyB* in the transconjugants harboring pE207. Moreover, transconjugants of *Pseudomonas* sp. strains D1A and D1B harboring pE207 were able to grow on MM with eugenol as the sole carbon source.

## Discussion

A 23-kb *Eco*RI fragment (E230) cloned from genomic DNA of *Pseudomonas* sp. strain HR199 was found to encode the proteins that are involved in the degradation of eugenol. In a recent study, genes *vdh*, *vanA*, and *vanB* encoding vanillin dehydrogenase and α- and β-subunits of vanillate-*O*-demethylase, respectively, which are responsible for the conversion of vanillin to protocatechuate, have been identified on this fragment (Priefert et al. 1997). The ORF identified upstream of the *vdh* gene (Priefert et al. 1997) was shown to encode an enoyl-CoA hydratase/aldolase that is also involved in eugenol and ferulic acid catabolism (Fig. 1; Gasson et al. 1998; Overhage et al. 1999b). In the present study, we describe the molecular characterization of a part of fragment E230 that is located between the aforementioned *vanAB* and *ech*/*vdh* gene regions (Fig. 2). A 1.8-kb *Kpn*I subfragment of E230 harboring gene *ehyA* complemented a nitrosoguanidine-induced mutant of *Pseudomonas* sp. strain HR199 that was unable to grow on eugenol as the sole carbon source. Genes *ehyA* and *ehyB*, which are involved in the first step of eugenol degradation, were found to encode the α- and β-subunits of a new eugenol hydroxylase. The function of these genes was confirmed by heterologous expression in *E. coli* and in various *Pseudomonas* strains. The expression of the genes conferred eugenol hydroxylase activity to the corresponding strains. These genes were located downstream of the *vanAB* genes that have been characterized recently (Priefert et al. 1997) and were separated from these only by two additional open reading frames with sequence similarities to glutathione-dependent formaldehyde dehydrogenases and γ-glutamylcysteine synthetases. Since formaldehyde is (in addition to protocatechuic acid) a product of the reaction catalyzed by vanillate-*O*-demethylase, the *fdh*-encoded formaldehyde dehydrogenase is most probably also involved in the degradation process and catalyzes the conversion of toxic formaldehyde to formiate, which is further metabolized. Whether the *gcs* gene product, which catalyzes the first step of the gluthatione biosynthesis, is involved in the degradation process remains unknown. Also the function

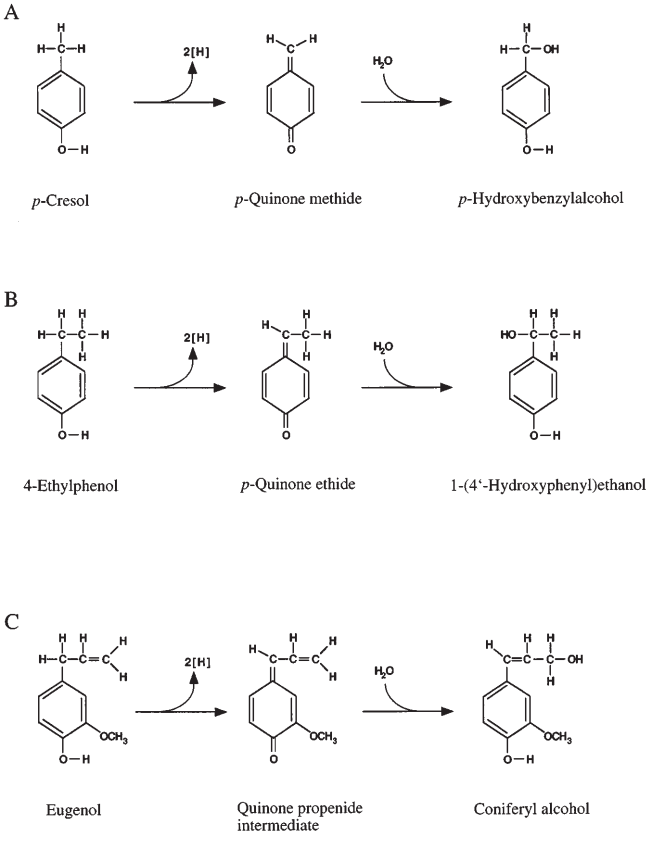


**Fig. 6** Comparison of the reactions catalyzed by *p*-cresol methylhydroxylase (**A**) and 4-ethylphenol methylenehydroxylase (**B**) with the proposed reaction mechanism for eugenol hydroxylase from *Pseudomonas* sp. strain HR199 (**C**)

of ORF2, which separates genes *ehyA* and *ehyB*, has to be elucidated. Therefore, the consequences of an inactivation of this open reading frame with respect to the eugenol hydroxylase activity will be studied in the future. Interestingly, genes *pchC* and *pchF* of the subunits of *p*-cresol methylhydroxylase from *Pseudomonas putida* are also separated by a stretch of approximately 700 nucleotides (Kim et al. 1994) comprising gene *pchX*, whose function is also unknown (Cronin et al. 1999). However, a comparison of the amino acid sequence deduced from ORF2 with that of the *pchX* gene product did not reveal any significant similarity. As described for the structural gene of the cytochrome *c* subunit of *p*-cresol methylhydroxylase from *Pseudomonas putida* (Kim et al. 1994), a signal sequence was also found for the *ehyA* gene product of *Pseudomonas* sp. strain HR199, indicating that eugenol hydroxylase is also located in the periplasmic space. On the other hand, no signal sequence was found for the flavoprotein subunit, as is also true for the *p*-cresol methylhydroxylase from *Pseudomonas putida* (Kim et al. 1994). The strong sequence similarities to the *p*-cresol methylhydroxylase from *Pseudomonas putida*, whose three-dimensional structure is known (Mathews et al. 1991), suggests analogous reaction mechanisms for *p*-cresol methylhydroxylase, 4-ethylphenol methylenehydroxylase (Reeve et al.

1989), and eugenol hydroxylase (Fig. 6); these mechanisms have also been proposed for the eugenol dehydrogenase of *Pseudomonas fluorescens* E118 (Furukawa et al. 1998) and for the vanillyl alcohol oxidase of *Penicillium simplicissimum* (Benen et al. 1998). To confirm these assumptions, future studies will focus on the investigation of the reaction mechanism. In this regard, the sequence data reported in the present study will allow the generation and investigation of site-specifically mutated forms of eugenol hydroxylase.

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