Cloning, expression, and characterization of a self-sufficient cytochrome P450 monooxygenase from *Rhodococcus ruber* DSM 44319

Luo Liu, Rolf D. Schmid, Vlada B. Urlacher*

Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

*To whom correspondence should be addressed: Tel.: +49-711-685-4523; Fax: +49-711-685-3196; E-mail: Vlada.Urlacher@itb.uni-stuttgart.de

Abstract

A new member of class IV of cytochrome P450 monooxygenases was identified in *Rhodococcus ruber* strain DSM 44319. As the genome of *Rhodococcus ruber* has not been sequenced, a P450-like gene fragment was amplified using degenerated primers. The flanking regions of the P450-like DNA fragment were identified by directional genome walking using PCR. The primary protein structure suggests a natural self-sufficient fusion protein consisting of a ferredoxin, flavin-containing reductase and P450 monooxygenase. The only flavin found within the enzyme was FMN. The enzyme was successfully expressed in *Escherichia coli* and purified and characterized. In the presence of NADPH, the P450 monooxygenase showed hydroxylation activity towards polycyclic aromatic hydrocarbons naphthalene, indene, acenaphthene, toluene, fluorene, *m*-xylene and ethyl benzene. The conversion of naphthalene, acenaphthene and fluorene resulted in respective ring monohydroxylated metabolites. Alkyl aromatics like toluene, *m*-xylene and ethyl benzene were hydroxylated exclusively at the side chains. The new enzyme's ability to oxidize such compounds makes it a potential candidate for biodegradation of pollutants and an attractive biocatalyst for synthesis.

Introduction

Cytochrome P450 monooxygenases are heme-containing proteins found in almost all living organisms (Werck-Reichhart and Feyereisen 2000). They catalyze a wide range of oxidation reactions, including aliphatic and aromatic hydroxylation, epoxidation of double bonds, oxidative phenolic coupling, dealkylation and multiple oxidation including carbon-carbon bond cleavage (Mansuy 1994; Cryle 2003).

Chemical oxidation reactions are often unspecific and lead to the formation of additional undesired by-products. They frequently require extremely high temperatures. Cytochrome P450 enzymes, which catalyze the stereo- and regiospecific hydroxylation of non-activated hydroxycarbons under mild conditions, have therefore become interesting candidates for industrial applications. Such biotransformations are especially useful in the food and pharmaceutical industries where high reaction selectivity on complex substrates is mandatory (Schmid et al. 2001). P450s can also be utilized in the bioremediation of pollutants, as these enzymes convert chemically inert compounds to more water-soluble, hydroxylated derivatives, which may be suitable substrates for many other enzymes (Harford-Cross et al. 2000; Li et al. 2001).

A large number of new chemical processes that involve an oxidation step, as well as the growing variety of toxic compounds that require treatment, means there is a need for new enzymes with new properties. In order to obtain enzymes with new features, several different approaches are being applied.

Nowadays, the generation of enzyme mutants by protein design (Bornscheuer and Pohl 2001; Bornscheuer 2002) or by directed evolution (Farinas et al. 2001; Seng Wong et al. 2004) is particularly popular. Both approaches have proven to be very useful in engineering P450 enzymes. Nevertheless, several drawbacks limit the broad application of these methods. Protein

design requires detailed structural information and the mechanistic data, but only a limited number of enzymes have been crystallized. Directed evolution can only be effective in combination with efficient and sensitive high-throughput screening assays. Unfortunately, it is not always possible to develop such assays. **An alternative strategy for identification new activities and selectivities is a classical microbiology screening of available microbial stains with consequent construction of gene-library or using the directional genome walking strategy. The latter approach can be applied to any gene if it contains one or more highly conserved regions, as in case of P450 monooxygenases the oxygen- and heme-binding regions.**

In this study we have screened several *Rhodococcus*, *Pseudomonas* and *Sphingomonas* strains for monooxygenase activity. A full-length cytochrome P450 monooxygenase gene was identified in the *Rhodococcus ruber* strain DSM 44319, cloned and heterologously expressed in *Escherichia coli*. **The protein sequence of the P450 monooxygenase reveled 93% identity to P450RhF from *Rhodococcus* sp. NCIMB 9784 (Roberts et al. 2002; Roberts et al. 2003) and 55% identity to CYP116 from *Rhodococcus erythropolis* NI86/21 (Nagy et al. 1995). However, during our work new enzymatic properties of this monooxygenase were identified, which have not been described for homologous enzymes.**

Materials and Methods

Chemicals and enzymes

Fluorene, 9-fluorenone and 9-fluorenol were purchased from Lancaster (UK). Acenaphthene, 7-ethoxycoumarin and 7-hydroxycoumarin were purchased from Sigma-Aldrich (Germany). All other chemicals were purchased from Fluka (Switzerland). Restriction endonucleases, T4 DNA ligase, and native Pfu DNA polymerase were obtained from Fermentas (Germany).

Bacterial strains

Rhodococcus rhodochrous NCIMB 11216, *Pseudomonas citronellolis* DSM 50332, *Pseudomonas fluorescens* DSM 50090, *Pseudomonas savastanoi* DSM 50267, *Sphingomonas echinoides* DSM 1805, *Rhodococcus erythropolis* DSM 43066 and *Rhodococcus ruber* DSM 44319 were used for activity screening. *Escherichia coli* BL21(DE3) strain was used for recombinant expression of the P450 monooxygenase. All strains were stored in 50% glycerol at -80°C and cultivated as recommended by the suppliers.

Biotransformation *in vivo*

50 ml of overnight culture of each bacterial strain were transferred into 50 ml Falcon tubes and centrifuged at 3220 × g and 4°C. The supernatant was discarded and the pellet was resuspended in 25 ml potassium phosphate buffer (50 mM, pH 7.4), supplemented with 1% glucose. The substrate was added to a final concentration of 5 mM. Aliquots of 1 ml were taken every hour for a period of 7 hours, and after approximately 16 hours. The samples were centrifuged and the supernatant was subsequently extracted with 250 µl toluene. The organic phase was then dried over anhydrous sodium sulfate. 1 µl sample was analyzed by GC-FID (HRGC MEGA 2 series, FISONS Instruments, Berverly, USA), using a 60 m ZB-5 column. For cyclohexane, the temperature program was 110°C for 5 min, the first temperature gradient of 5°C min^{-1} to 150°C, and a second temperature gradient of 10°C min^{-1} to 200°C. Under such conditions the retention times were: for cyclohexane 5.75 min; for cyclohexanol 9.41 min; for cyclohexanone 9.59 min.

PCR with Dynazyme^{TM} EXT

Isolation of genomic DNA was performed using a standard phenol/chloroform precipitation protocol as described by Sambrook and Russell (Sambrook 2000). DNA fragment was amplified

using degenerated primers (s. below). Dynazyme^{TM} EXT was used according to the manufacturer's protocol in order to obtain a high yield and low nonspecific products during PCR on a GC-rich template (up to 70% GC content). If the PCR product was later used for TA-cloning, a 20 min extension at 72°C was included in order to ensure full-length PCR products and 3' adenylation.

Amplification of a cytochrome P450-like gene fragment using degenerated primers

In order to identify a cytochrome P450-like gene within genomic DNA of a *Rhodococcus* strain, two degenerated primers, which are specific for the most conserved regions, were designed on the basis of the sequence alignment of CYP116 (Nagy et al. 1995) and P450RhF (Roberts et al. 2002). Forward primer: 5'-CTACTGGGTSGTCACSCGSTACGA-3'; reverse primer: 5'-GCAYTCCTCGAYGGCSTTGGGGAT-3'. The PCR products were analyzed by agarose gel (1%) electrophoresis. The DNA band corresponding to the expected length of 740 bp was excised and purified using the QIAquick® gel extraction kit from Qiagen (Germany). For sequencing, PCR products were cloned using the Topo® TA cloning kit (Invitrogen, USA) according to the manufacturer's protocol. The insert was sequenced using universal M13 forward and reverse primers. The sequences were aligned to those of CYP116 and P450RhF using ClustalX (www-igbmc.u-strasbg.fr/BioInfo/ClustalX).

Directional genome walking

In order to explore the flanking genomic sequences of the amplified 740 bp fragment, we employed a method called "directional genome walking using PCR" described by Mishra *et al.* (Mishra et al. 2002), using one biotinylated primer, designed on the basis of the identified P450-like sequence, and a non-biotinylated nested and four walking primers.

The biotinylated specific primer: Biotin-5'-TCGAGGGCGATGGCCGGTGA-3'

Non-biotinylated nested primer 1: 5'-AACACGAGGTTGTCGCGGAA-3'

Walking primers expected to anneal in the flanking regions up- and downstream of the amplified 740 bp fragment were taken from the original publication (Mishra et al. 2002). Primary PCRs were performed with genomic DNA, biotinylated primer and each of the four walking primers separately. The PCR conditions were as described before.

Streptavadin-coupled magnetic beads (Roche, Switzerland) were used as described in the manufacturer's protocol in order to remove most unspecific PCR products. The second nested PCR was applied to minimize the amount of unspecific products (Mishra et al. 2002).

The next primers were subsequently designed for the next steps of genomic walking. After five walking steps the total sequence of the gene was completed successfully.

Creation of an expression construct

After successful genomic walking, a full-length gene encoding P450 protein was amplified from genomic DNA by PCR using the following primers: 5'-ctgGAATTCATGAGTGCATCAGTTCCGGCGT-3' and 5'-catcAAGCTTTCAGAGTCGCAGGGCCA-3'. The *Eco*RI and *Hin*dIII restriction endonuclease sites in the primer sequences are underlined. The PCR product was isolated and digested with *Eco*RI and *Hin*dIII restriction endonucleases, cloned into the pET28a(+) vector, and expressed in *E. coli* BL21(DE3) cells. The sequence of the insert DNA was subsequently confirmed by sequencing.

Protein expression and purification

E. coli BL21(DE3) containing the expression construct was grown in LB medium (4 × 400 ml in 2 liter shake flask), supplemented with 30 μg ml^{-1} kanamycin, at 30°C and 120 rpm. Expression was induced with 0.5 mM IPTG and cells were incubated overnight at 25°C. The cells were pelleted at 10,000 × g for 20 min and disrupted by sonification on ice using a Sonifier 250 (Branson, Germany). After centrifugation cell lysate was loaded onto a Nickel sepharose column, and equilibrated with loading buffer (50 mM potassium phosphate, 300 mM NaCl, pH 7.5). After purification with 50 mM imidazole, the protein was eluted and dialyzed against potassium phosphate buffer, pH 7.4 using a Vivaspin 15R membrane of 30,000 (Vivasience, Germany). Finally, the sample was concentrated threefold and stored at -20°C with 10% glycerol until further use. The sample was analyzed on 12.5% SDS-PAGE and the protein visualized with Coomassie Brilliant Blue. The concentration of P450 protein was determined from the CO difference spectrum as described elsewhere (Omura and Sato 1964). The purity of the P450 protein was determined densitometrically by Scion Image Beta 4.0.2 (Scion Corporation, USA).

Spectroscopic characterization

Absorption spectra of the purified P450 monooxygenases were recorded with a spectrophotometer (Ultraspec 3000, Pharmacia Biotech, UK) in glass cuvettes with a path length of 1 cm. The protein concentration was approximately 1 μM. The P450 monooxygenase was reduced by 50 μM NADPH or sodium dithonite.

Chromatographic determination of flavin

To release a flavin compound, P450 was denatured at 45°C for 20 min and subsequently centrifuged at 22,000 × g for 30 min. 2 μl supernatant were analyzed by thin-layer chromatography (TLC) on silica gel plates (Kieselgel 60 F_{254}, Merck, Germany). The solvent

system comprised *n*-butanol, acetic acid, and water (5:2:3, vol:vol:vol) (Gliszczynska and Koziolowa 1998). FMN (riboflavin 5'-monophosphate) and FAD (flavin adenine dinucleotide) were used as controls. TLC plates were visualized by exposure to UV light (280 nm). The concentration of FMN in the supernatant was determined from its fluorescence (excitation: 470 nm; emission: 510 nm) in a 96-well plate (FLUOstar BMG, Germany) and calculated by using a series of FMN standards. The molar ratio of FMN to protein was expressed as FMN concentration against protein concentration.

Reductase domain activity determination

Tests for reductase domain-dependent electron transfer to exogenous electron acceptor cytochrome c were performed as described by Gustafsson *et al*. (Gustafsson et al. 2004). The K_M and k_{cat} values were determined graphically using standard methods.

Biotransfomation using isolated enzyme and GC-MS analysis

For the 7-ethoxycoumarin biotransformation, 190 µl mixtures containing 50 mM potassium phosphate buffer and 1 µM of purified P450 were placed in 96-well plates. 7-Ethoxycoumarin was added from 250 mM stock solution in DMSO to a final concentration of 1.25 mM. The mixture was incubated at room temperature for 5 min**.** The addition of 10 µl NADPH (6.7 mM) started the reaction. **The same mixtures without NADPH or enzyme were used as negative controls.** The 96-well plates were placed immediately in a microtiter plate reader (FLUOstar, BMG, Germany) and a fluorescence intensity of 7-hydroxycoumarin was measured (excitation at 405 nm, emission at 460 nm). The product formation rate was calculated by using a series of 7-hydroxcoumarin standards with different concentrations.

For the biotransformation of naphthalene, indene, acenaphthene, toluene and fluorene, 900 µl mixtures containing 50 mM potassium phosphate buffer, 1 µM P450, 1.25 mM substrate were incubated at room temperature for 5 min. Reactions were started by the addition of 100 µl NADPH (6.7 mM). **Reaction mixtures without NADPH or enzyme were used as negative controls.** After 2 hours incubation at 25°C the mixture was extracted with 350 µl diethyl ether. The organic phase was subsequently dried over anhydrous magnesium sulfate. 1 µl sample was analyzed by GC-MS (GCMS QP2010, Shimadzu, Japan). The calibration curves were determined by measuring different concentrations of the expected products. The temperature programs for the various chemicals were: naphthalene, 100-275°C (10°C min^{-1})*;* acenaphthene and fluorene, 150°C (1 min) – 150-270°C (20°C min^{-1}); toluene, 70°C (2 min) – 70-250°C (15°C min^{-1}); indene, 100°C (5 min) – 100-250°C (15°C min^{-1}); cyclohexane, 50°C (5 min) – 50-250°C (20°C min^{-1}); alpha-pinene, 90°C (5 min) – 90-250°C (15°C min^{-1}); terpineol, 100°C (1 min) – 100-250°C (20°C min^{-1}).

Biotransformation using recombinant *E. coli* cells

To determine activity towards *m*-xylene and ethyl benzene, the *E. coli* BL21(DE3) expression strain carrying the P450 gene was used as a catalyst. **The same *E. coli* strain containing the empty pET28a(+) vector was used as a negative control**. The substrate was added to a final concentration of 2 mM in 200 ml overnight induced culture. Cells were incubated for 24 hours at 25°C and 120 rpm. The product formed was extracted with 50 ml dichloromethane and concentrated by evaporation to 1 ml. 1 µl sample was analyzed by GC-MS. The temperature program for *m*-xylene and ethyl benzene was: 70°C (2 min) – 70-250°C (15°C min^{-1}).

Nucleotide sequence accession number

The nucleotide sequence reported in this paper was deposited in the GenBank database with the accession number AY957485.

Results

Screening for strains with monooxygenase activity

In this study we were interested in finding new cytochrome P450 activities from bacterial sources. Bacterial P450 enzymes are in most cases not membrane-associated and exhibit relatively high stability (Werck-Reichhart and Feyereisen 2000). **Seven bacterial strains of the genera *Rhodococcus*, *Pseudomonas* and *Sphingomonas* were screened for their hydroxylation activity towards cyclohexane. Cyclohexane was chosen due to its rather low toxicity and industrial importance. Microorganisms of genus *Rhodococcus* are widely spread in the nature and known to transform a huge range of xenobiotics (Finnerty 1992). The typical *Pseudomonas* bacteria in nature might be found in a biofilm; they are ubiquitous in soil and water and have been shown to grow in the presence of alkanes (van Beilen et al. 1994). Due to their biodegradative and biosynthetic capabilities, several *Sphingomonas* stains have also been tested in this work. Besides that several cytochrome P450 monooxygenases have already been identified and isolated from these three genera (Hedegaard and Gunsalus 1965; Tyson et al. 1972; Ropp et al. 1993; Fruetel et al. 1994).**

GC analysis of the products of the biotransformations revealed only two *Rhodococcus* strains with activity towards cyclohexane, namely *Rhodococcus erythropolis* DSM 43066 and *Rhodococcus ruber* DSM 44319. **Cyclohexanone was the only product detected, and we therefore assumed that the microorganism can contain cyclohexane hydroxylase, which hydroxylates cyclohexane to cyclohexanol and further to cyclohexanone; or probably, additionaly a dehydrogenase, which oxidizes cyclohexanol to cyclohexanone.** The

Pseudomonas citronellolis DSM 50332 and *Pseudomonas fluorescens* DSM 50090 strains did not display any activity towards cyclohexane. *Pseudomonas savastanoi* DSM 50267, *Sphingomonas echinoides* DSM 1805 and *Rhodococcus rhodochrous* NCIMB 11216 strains did not survive in the presence of cyclohexane.

Gene isolation

Genomic DNA was isolated from two active strains: *Rhodococcus erythropolis* DSM 43066 and *Rhodococcus ruber* DSM 44319. Using degenerated PCR primers a P450-like DNA fragment of about 740 bp was amplified from genomic DNA of *Rhodococcus ruber* DSM 44319 strain but not from *Rhodococcus erythropolis* DSM 43066. Sequencing and sequence alignment revealed the nucleotide sequence identity between the identified DNA fragment and the P450 monooxygenase domain of P450RhF from *Rhodococcus* sp. NCIMB 9784 of about 96%. The sequence identity between the DNA fragment from *Rhodococcus ruber* DSM 44319 and CYP116 from *Rhodococcus* sp. NI86/21 was about 66%. This high homology enables assignment of the new enzyme to the CYP116 family.

The entire P450 gene was identified by directional genome walking using PCR (Mishra et al. 2002). The P450 sequence obtained was translated into a protein of 771 amino acids. A BLAST search using the BLOSUM62 standard scoring matrices (Altschul 1991) (www.ncbi.nlm.nih.gov/BLAST) revealed that this protein is a natural fusion protein consisting of a heme domain, a flavin-reductase domain and a ferredoxin domain. This protein organization is the same as in P450RhF from *Rhodococcus* sp. NCIMB 9784 (Roberts et al. 2003). The overall nucleotide and amino acids sequence identity between the new P450 enzyme and P450RhF is > 90%. De Mot and Parret identified four putative fusion P450 enzymes of this type also in three

pathogenic *Burkholderia* species and in a heavy metal-resistant bacterium *R. metallidurans* (De Mot and Parret 2002).

Recombinant expression and protein purification

P450 gene from *Rhodococcus ruber* DSM 44319 was cloned in the pET28a(+) expression vector. The resulting expression product contained 807 amino acids including a His_6-tag at the N-terminus. Yield of active P450 monooxygenase, calculated from a CO-difference spectrum, amounted to 18.6 mg l^{-1}. His_6-tag allowed for easy high-performance one-step purification of the fusion protein by immobilized metal affinity chromatography (purity 85%, recovery 73%, Fig. 1). The main band in lane 4 corresponds to the expected expression product at 89,000 Da that is identical to the molecular weight estimated from the protein sequence.

Spectroscopic characterization of the enzyme

The absorption spectrum of oxidized protein showed a heme Soret band at 418 nm, which is typical for cytochrome P450 enzymes. The broad absorption shoulder between 450 and 510 nm indicates presence of flavins. The addition of NADPH led to a decrease of this shoulder due to reduction of the oxidized flavin component. The addition of carbon monoxide to the dithionite-reduced protein resulted in a Soret band shift from 418 nm to 449 nm as is expected for a cytochrome P450 enzyme.

Identification of the flavin cofactor bound to the reductase was carried out using thin-layer chromatography (TLC). The analyzed sample had the same R_f-value of 0.41 as an authentic FMN, while FAD showed a different R_f-value of 0.24. No FAD was detected in the sample. The concentration of released FMN from enzyme was 0.12 mM as estimated from fluorescence measurements. Protein concentration in the analyzed sample was 0.11 mM; the molecular ratio

between FMN and the protein is therefore 1.1:1, which is an indication that each P450 monooxygenase molecule contains one FMN molecule.

In order to determine the reductase activity of the enzyme, cytochrome c was used as exogenous electron acceptor. The result showed that the new P450 monooxygenase exhibited a preference for NADPH over NADH during this reduction, same as P450RhF. The k_{cat} value for NADPH as an electron donor is more than twice that for NADH, and the K_M value for NADPH is 30-fold lower than that for NADH (Table 1).

Determination of substrate spectra and hydroxylation activity

After isolation and purification P450 from *Rhodococcus ruber* DSM 44319 was tested for its oxidation activity towards several different chemical compounds. 7-Ethoxycoumarin was selected because the highly homologous P450RhF catalyzes the O-dealkylation, leading to 7-hydroxycoumarin. Both 7-ethoxycoumarin and 7-hydroxycoumarin are strongly fluorescent and can be separately identified by fluorescence spectroscopy. The rate of O-dealkylation of 7-ethoxycoumarin by the P450 monooxygenase is given in Table 2.

As a toluene-degrading biofilm, being used for microbiological water purification consists of different *Rhodococcus* strains, toluene was also selected as a substrate. Furthermore, the polycyclic aromatic hydrocarbons naphthalene, acenaphthene, and fluorene as well as indene were chosen. Aromatic hydrocarbons were oxidized by the P450 monooxygenase from *Rhodococcus ruber* DSM 44319 far slower than 7-ethoxycoumarin. The products derived from the corresponding substrates are summarized in Figure 2. Naphthalene was oxidized regioselectively to 1-naphthol (RT, 13.03 min, Table 2). Acenaphthene was oxidized to 1-acenaphthenol (RT, 3.99 min). A single product of fluorene oxidation was identified as 9-fluorenol (RT, 4.43 min). The oxidation of indene by the P450 monooxygenase resulted in

indenol (RT, 7.89 min). Interestingly, oxidation of toluene by P450 from *Rh. ruber* resulted in benzyl alcohol (retention time, RT, 5.73 min). The activity was approximately threefold lower than the activity towards 7-ethoxycoumarin.

In order to confirm the selectivity of the new P450 monooxygenase, two other alkyl aromatic hydrocarbons were tested - *m*-xylene and ethyl benzene (Table 2 and Fig. 2). **As activity towards these compounds was low, whole-cell oxidation using recombinant *E. coli* cells producing the P450 enzyme was performed in order to produce a sufficient amount of the hydroxylated products**. Only 3-methylbenzyl (RT, 6.78 min) alcohol was detected as a product of *m*-xylene oxidation. In the case of ethyl benzene, however, two different products were identified: 1-phenylethyl alcohol (RT, 5.92 min) and 2-phenylethyl alcohol (RT, 6.62 min). No product was detected in control culture cells with empty vector. Both substrates as well as toluene were hydroxylated exclusively at the alkyl chain and no ring oxidation was observed. Such selectivity towards the alkyl chain of aromatics is remarkable and might be applied in technical processes, as chemical oxidation is complicated and requires harsh reaction conditions.

No hydroxylation activity towards α-pinene, terpeneol, ethoxyresorufin was detected. Remarkably, no hydroxylation activity towards cyclohexane was detected with the purified enzyme. One may speculate whether or not another P450 monooxygenase is responsible for the hydroxylation of cyclohexane in this *Rhodococcus* strain.

Discussion

In this paper, we have reported the identification and characterization of a new cytochrome P450 monooxygenase from *Rhodococcus ruber* DSM 44319, using a genomic walking method. This approach facilitates the cloning, characterization, and expression of new cytochrome P450 genes without difficult steps of protein purification and sequencing. The complicated and time-

consuming probing of a genomic DNA library and the subsequent sequencing of positive clones can also be avoided.

A P450 gene from *Rhodococcus ruber* DSM 44319 was successfully cloned, expressed in *Escherichia coli,* and the enzyme was purified using immobilized metal affinity chromatography. Thin layer chromatography detected only FMN within the reductase domain. FMN-containing reductases are usually involved in the electron transport from NADH to dioxygenases. Thus, these results as well as a high sequence homology (93% identity) to P450RhF from a *Rhodococcus* sp. NCIMB 9784 indicate that a new NADPH dependent natural fusion protein from *Rhodococcus ruber* belongs to class IV of cytochrome P450 monooxygenases postulated by Roberts *et al.* (Roberts et al. 2003).

Interestingly, the most known bacterial fusion P450 monooxygenases such as CYP102A1, CYP120A2 and CYP102A3 from *Bacilli* (Narhi and Fulco 1986; Gustafsson et al. 2004), as well as the eukaryotic membrane-bound CYP505 from *Fusarium oxysporum* (Nakayama et al. 1996) contain a diflavin (FAD and FMN) P450 reductase (belonging to class II). All fusion P450 monooxygenase systems are self-sufficient, as they do not require any additional proteins for an electron transfer from NADPH to the heme iron and therefore good candidates for industrial applications.

A highly homologues P450RhF from *Rhodococcus* sp. NCIMB 9784 has been reported to be only able to catalyze dealkylation of 7-ethoxycoumarin from a number of substances, which were not specified (Roberts et al. 2002). Due to high homology to P450RhF, the P450 monooxygenase from *Rhodococcus ruber* exhibits also an NADPH-dependence and also catalyzes dealkylation of 7-ethoxycoumarin. However, activity of P450RhF (4.9 min^{-1}) (Roberts et al. 2003) is higher than that of P450 from *Rhodococcus ruber* (0.917 min^{-1}). The P450 monooxygenase from *Rhodococcus ruber* was found to hydroxylate besides that a broad

range of aromatics. All oxidized substrates contain at least one benzene ring and a functional group (Fig. 2). We suggest that the benzene ring is required for the recognition by the P450 monooxygenase. Nevertheless benzene itself is not a suitable substrate for this enzyme. Benzene may be excessively small for the enzymes binding pocket and cannot be positioned close enough to the heme group. Toluene however was oxidized only at the methyl group and not on the benzene ring. The obtained results indicated that polycyclic aromatic hydrocarbons with three rings were hydroxylated by the P450 monooxygenase much slower than naphthalene, which consists of only two aromatic rings (Table 2). The PAHs are ubiquitous pollutants and harmful (Shuttleworth and Cerniglia 1995). Hydroxylation of PAHs enables its biodegradation (Li et al. 2001). On the one hand, these properties open up new possibilities for optimizing bioremediation processes. On the other hand, the ability to hydroxylate the alkyl chain of aromatics makes this enzyme a useful biocatalyst for synthesis.

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References

Altschul SF (1991) Amino acid substitution matrices from an information theoretic perspective. J Mol Biol 219:555-565

Bornscheuer UT (2002) Methods to increase enantioselectivity of lipases and esterases. Curr Opin Biotechnol 13:543-547

Bornscheuer UT, Pohl M (2001) Improved biocatalysts by directed evolution and rational protein design. Curr Opin Chem Biol 5:137-143

Cryle MJ, Stok J. E. and De Voss J. J. (2003) Reactions catalyzed by bacterial cytochromes P450. Aust J Chem 56:749-762

De Mot R, Parret AH (2002) A novel class of self-sufficient cytochrome P450 monooxygenases in prokaryotes. Trends Microbiol 10:502-508

Farinas ET, Bulter T, Arnold FH (2001) Directed enzyme evolution. Curr Opin Biotechnol 12:545-551

Finnerty WR (1992) The biology and genetics of the genus *Rhodococcus*. Annu Rev Microbiol 46:193-218

Fruetel JA, Mackman RL, Peterson JA, Ortiz de Montellano PR (1994) Relationship of active site topology to substrate specificity for cytochrome P450terp (CYP108). J Biol Chem 269:28815-28821

Gliszczynska A, Koziolowa A (1998) Chromatographic determination of flavin derivatives in baker's yeast. J Chromatogr A 822:59-66

Gustafsson MC, Roitel O, Marshall KR, Noble MA, Chapman SK, Pessegueiro A, Fulco AJ, Cheesman MR, von Wachenfeldt C, Munro AW (2004) Expression, purification, and characterization of *Bacillus subtilis* cytochromes P450 CYP102A2 and CYP102A3: flavocytochrome homologues of P450 BM3 from Bacillus megaterium. Biochemistry 43:5474-5487

Harford-Cross CF, Carmichael AB, Allan FK, England PA, Rouch DA, Wong LL (2000) Protein engineering of cytochrome p450(cam) (CYP101) for the oxidation of polycyclic aromatic hydrocarbons. Protein Eng 13:121-128

Hedegaard J, Gunsalus IC (1965) Mixed function oxidation. IV. An induced methylene hydroxylase in camphor oxidation. J Biol Chem 240:4038-4043

Li QS, Ogawa J, Schmid RD, Shimizu S (2001) Engineering cytochrome P450 BM-3 for oxidation of polycyclic aromatic hydrocarbons. Appl Environ Microbiol 67:5735-5739

Mansuy D (1994) Cytochromes P-450 and Model Systems - Great Diversity of Catalyzed-Reactions. Pure and Applied Chemistry 66:737-744

Mishra RN, Singla-Pareek SL, Nair S, Sopory SK, Reddy MK (2002) Directional genome walking using PCR. Biotechniques 33:830-832, 834

Nagy I, Schoofs G, Compernolle F, Proost P, Vanderleyden J, de Mot R (1995) Degradation of the thiocarbamate herbicide EPTC (S-ethyl dipropylcarbamothioate) and biosafening by *Rhodococcus* sp. strain NI86/21 involve an inducible cytochrome P-450 system and aldehyde dehydrogenase. J Bacteriol 177:676-687

Nakayama N, Takemae A, Shoun H (1996) Cytochrome P450foxy, a catalytically self-sufficient fatty acid hydroxylase of the fungus *Fusarium oxysporum*. J Biochem (Tokyo) 119:435-440

Narhi LO, Fulco AJ (1986) Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus megaterium*. J Biol Chem 261:7160-7169

Omura T, Sato R (1964) The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. J Biol Chem 239:2370-2378

Roberts GA, Celik A, Hunter DJ, Ost TW, White JH, Chapman SK, Turner NJ, Flitsch SL (2003) A self-sufficient cytochrome p450 with a primary structural organization that includes a flavin domain and a [2Fe-2S] redox center. J Biol Chem 278:48914-48920

Roberts GA, Grogan G, Greter A, Flitsch SL, Turner NJ (2002) Identification of a new class of cytochrome P450 from a *Rhodococcus* sp. J Bacteriol 184:3898-3908

Ropp JD, Gunsalus IC, Sligar SG (1993) Cloning and expression of a member of a new cytochrome P-450 family: cytochrome P-450lin (CYP111) from *Pseudomonas incognita*. J Bacteriol 175:6028-6037

Sambrook J, Russell, DW (2000) Molecular Cloning, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B (2001) Industrial biocatalysis today and tomorrow. Nature 409:258-268

Seng Wong T, Arnold FH, Schwaneberg U (2004) Laboratory evolution of cytochrome p450 BM-3 monooxygenase for organic cosolvents. Biotechnol Bioeng 85:351-358

Shuttleworth KL, Cerniglia CE (1995) Environmental aspects of PAH biodegradation. Appl Biochem Biotechnol 54:291-302

Tyson CA, Lipscomb JD, Gunsalus IC (1972) The role of putidaredoxin and P450 cam in methylene hydroxylation. J Biol Chem 247:5777-5784

van Beilen JB, Wubbolts MG, Witholt B (1994) Genetics of alkane oxidation by *Pseudomonas oleovorans*. Biodegradation 5:161-174

Werck-Reichhart D, Feyereisen R (2000) Cytochromes P450: a success story. Genome Biol 1:REVIEWS3003

Figure legends

Fig. 1: Purification of P450 from *Rhodococcus ruber* DSM 44319 visualized on SDS-PAGE. The overexpressed protein is visible at 89 kDa after induction with IPTG. Lanes: 1- cell extracts; 2- column flow-through fraction; 3- washing fraction; 4- purified enzyme; M- molecular weight standard. The arrow shows the recombinant protein

Fig. 2: Reactions catalyzed by the P450 monooxygenase from *Rhodococcus ruber* DSM 44319

Figure 1:

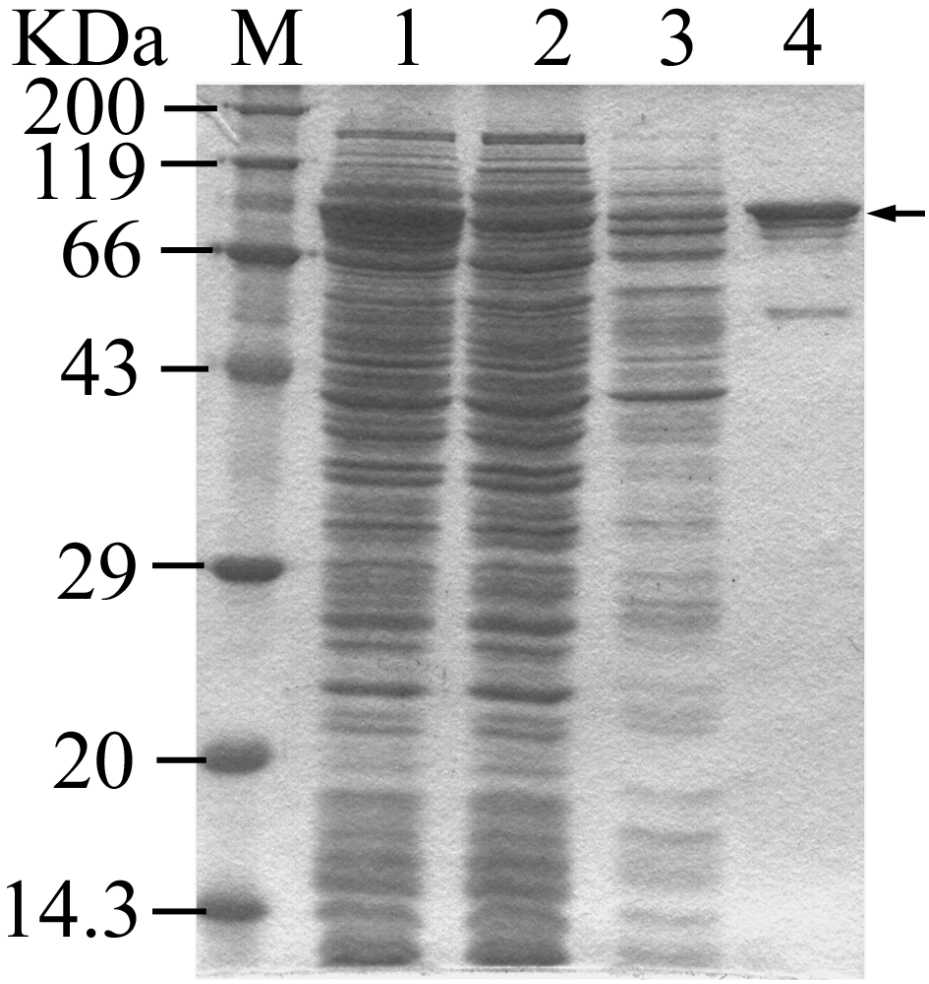
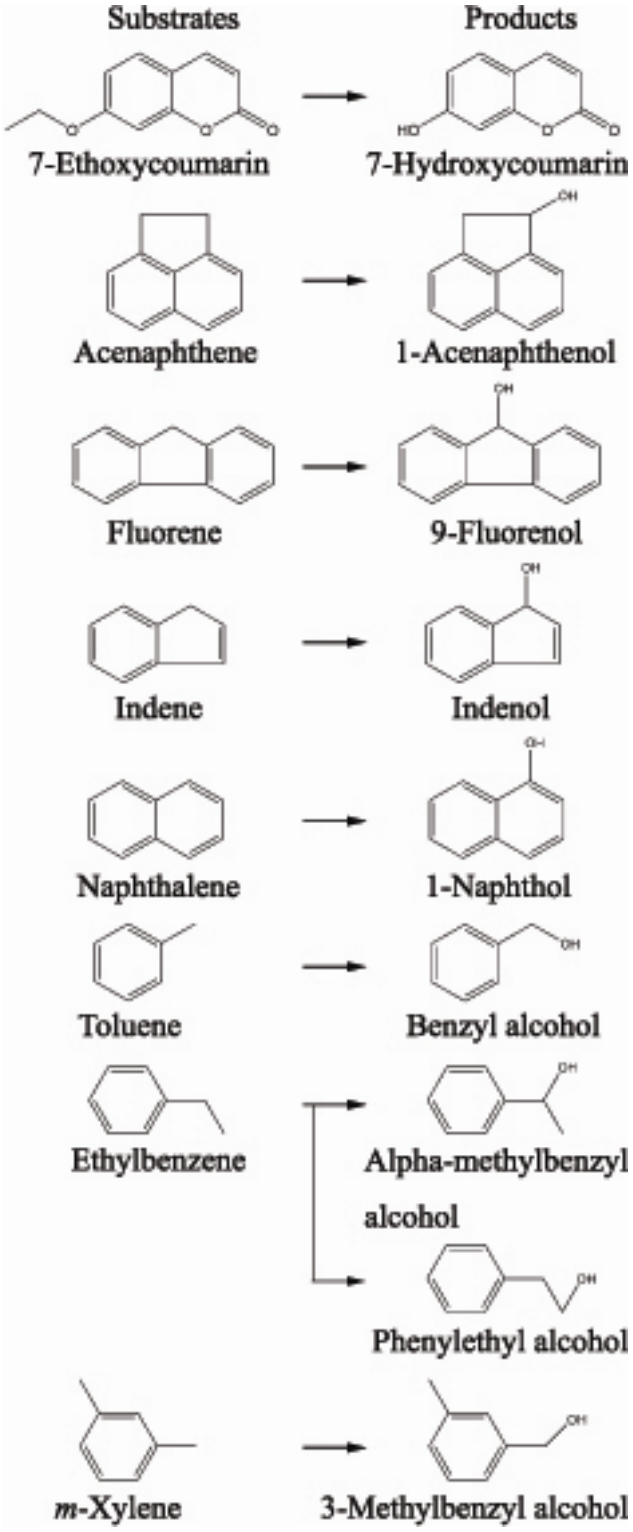


Figure 2:


Substrates
Products
7-Ethoxycoumarin
7-Hydroxycoumarin
Acenaphthene
1-Acenaphthenol
Fluorene
9-Fluorenol
Indene
Indenol
Naphthalene
1-Naphthol
Toluene
Benzyl alcohol
Ethylbenzene
Alpha-methylbenzyl alcohol
Phenylethyl alcohol
m-Xylene
3-Methylbenzyl alcohol

Tables

Table 1: Kinetic parameters of the P450 monooxygenase from *Rhodococcus ruber* DSM 44319 with cytochrome c as electron acceptor.

cofactor	K_M (µM)	v_{max} (µM min^{-1})	k_{cat} (min^{-1})
NADPH	3.4 ± 0.8	15.3 ± 3.0	764.8 ± 144.5
NADH	126.2 ± 11	7.2 ± 0.3	359.8 ± 11.5

Table 2: Substrates, products, and product formation rates of the P450 monooxygenase from *Rhodococcus ruber* DSM 44319.

Substrate	Product	Product formation rate (nmol x nmol P450 $^{-1}$ x min^{-1})
7-Ethoxycoumarin	7-Hydroxycoumarin	0.917 ± 0.05
Acenaphthene	1-Acenaphthenol	0.079 ± 0.01
Fluorene	9-Fluorenol	0.04 ± 0.005
Naphthalene	1-Naphthol	0.106 ± 0.01
Indene	1-Indenol	n. d.
Toluene	Benzyl alcohol	0.301 ± 0.01
Ethyl benzene	1-Phenylethyl alcohol	n. d.
	2-Phenylethyl alcohol	n. d.
m-Xylene	3-Methylbenzyl alcohol	n. d.

n. d. - not determined