

Tricistronic overexpression of cytochrome P450_{cam}, putidaredoxin, and putidaredoxin reductase provides a useful cell-based catalytic system

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Abstract The catalytic turnover of cytochrome P450_{cam} from *Pseudomonas putida* requires two auxiliary reduction partners, putidaredoxin (Pd) and putidaredoxin reductase (PdR). We report the functional expression in *Escherichia coli* of tricistronic constructs consisting of P450_{cam} encoded by the first cistron and the auxiliary proteins, Pd and PdR by the second and the third. Transformed bacterial whole cells efficiently oxidized (1R)-(+)-camphor to 5-exo-hydroxycamphor and, interestingly, limonene to (–)-perillyl alcohol. These bioengineered *E. coli* cells possess a heterologous self-sufficient P450 catalytic system that may have advantages in terms of low cost and high yield for the production of fine chemicals.

Keywords Camphor · Limonene · P450 · Tricistronic expression

Introduction

Cytochrome P450 (P450) enzymes constitute a superfamily of hemoproteins that catalyze mixed-function oxidation reactions in the metabolism of xenobiotic chemicals (Ortiz de Montellano 2005). P450 enzymes are found in nearly all life forms from bacteria to human (Guengerich 2001). P450_{cam} (CYP101) was the first P450 for which an X-ray crystal structure was determined (Poulos et al. 1982). The function of P450_{cam} in *Pseudomonas putida* is the oxidation of camphor to 5-*exo*-hydroxycamphor, which makes it possible for the bacteria to grow on camphor as a sole carbon source (Trudgill et al. 1966). P450_{cam} requires two auxiliary proteins: the flavoprotein putidaredoxin reductase (PdR) and the iron-sulfur protein putidaredoxin (Pd) for catalytic turnover (Katagiri et al. 1968). As a prototypic model of P450 for most mechanistic and structural studies, the wealth of information available on P450_{cam} makes it an attractive candidate for the development of a self-sufficient catalytic system (Sibbesen et al. 1996). In this study, we constructed a tricistronic expression system including P450_{cam}, Pd, and PdR in *Escherichia coli* and demonstrated its catalytic activity towards camphor and limonene. This system may be applicable in the industrial production of fine chemicals.

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Materials and methods

Materials

(1R)-(+)-Camphor, *L*-limonene, perillyl alcohol, and Nitroblue Tetrazolium were purchased from Aldrich Chemical Co. (Milwaukee, WI). Other chemicals were of the highest grade commercially available. The plasmids containing cDNA clones for Pd and PdR were kindly provided by Prof. Julian A. Peterson (University of Texas Southwestern Medical Center, Dallas, TX).

Construction of tricistronic expression vector

The open reading frames of Pd and PdR containing the Shine-Dalgarno (SD) ribosome binding sequences and *Hind*III, *Nhe*I and *Xba*I restriction enzyme sites were amplified using PCR as described in Fig. 1. The amplified PCR fragments were purified and cloned into the pCW(ori⁺) P450_{cam} vector containing *Hind*III and *Xba*I restriction enzyme sites at the C-terminus of the

P450 open reading frame (Sibbesen et al. 1998). The ligation mixture was transformed into *E. coli* DH5 α competent cells (Invitrogen, Carlsbad, CA) and then the plasmid DNA was purified using a Promega Wizard miniprep kit (Promega, Madison, WI). The recovered plasmid DNA was sequenced and verified for construction of the tricistronic system.

Tricistronic operon expression in *E. coli*

The constructed tricistronic DNA plasmid was transformed into *E. coli* DH5 α and then plated on LB medium/ampicillin (100 μ g/ml). A single colony was picked and transferred to single 5 ml of LB liquid medium/ampicillin. After overnight incubation at 37°C, the starter culture was diluted into 1 l of TB expression medium/ampicillin (100 μ g/ml) containing 1 mM IPTG, 0.5 mM δ -ALA, and 1 mM thiamine. The expression culture was grown at 37°C for 3 h and then at 24°C with shaking at 200 rpm for 24 h.

Enzyme assays

After the expression in TB medium, the *E. coli* cells were harvested and resuspended with M9 medium including 0.4% glucose as a carbon source. The reactions were performed at room temperature by addition of camphor or limonene (final 1 mM each) to the resuspended whole cells. The reaction products were analyzed by GC-MS after extraction with methylene dichloride.

Nitroblue Tetrazolium (NBT) assays

The reductase activity of PdR in *E. coli* whole cells was analyzed using NBT assays with some modification. The increase in absorbance at 535 nm was determined after adding 1 mM NADH. Calculation of the specific activity for PdR assumed an absorption coefficient of 12.8 mM⁻¹cm⁻¹ (Murphy et al. 1998).

GC-MS analyses of camphor and limonene and their metabolites

Analyses were performed on an Agilent 6850 gas chromatograph coupled to an Agilent 5973 Network Mass Selective Detector in the flame ionization mode. Helium was used as the carrier gas. The GC

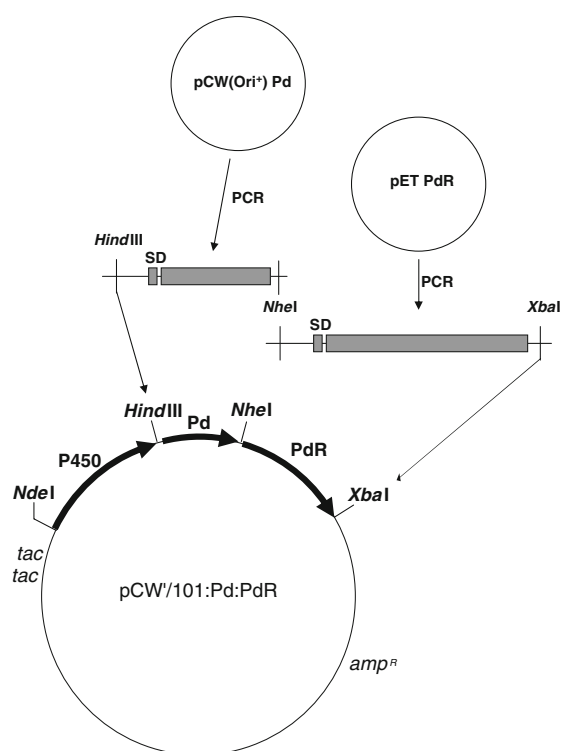


Fig. 1 Generalized construction scheme for CYP101: Pd: PdR tricistronic expression plasmids

was equipped with an HP-5MS cross-linked 5% PH ME Siloxane capillary column (30 m, 0.25 mm i.d., 0.25 μ m film thickness). The temperature program was 70°C for 1 min and then 70–280°C at 15°C/min or 70°C for 5 min, 70–160°C at 5°C/min, and then 160–280°C at 20°C/min. The temperatures were 250°C for the injection port and 280°C for the detector. A sample of 3 μ l was injected in a 10:1 split ratio onto the column. Identification of camphor, 5-exo-hydroxycamphor, limonene, and (–)-perillyl alcohol was done by co-chromatography and mass spectrometric comparison with authentic standards.

Results

Tricistronic operon expression in *E. coli*

In order to establish that P450_{cam}, Pd, and PdR were being overexpressed from the plasmid-borne operons, the soluble fraction was isolated and subjected to SDS-PAGE/Coomassie staining (Fig. 2). Bands with apparent molecular masses of ~47 kDa (P450), 45 kDa (PdR), and 11 kDa (Pd) were present. The identity of these proteins was confirmed by co-migration with previously purified proteins. The control experiment without IPTG induction showed that there was no expression of proteins with the corresponding sizes (data not shown). CO difference

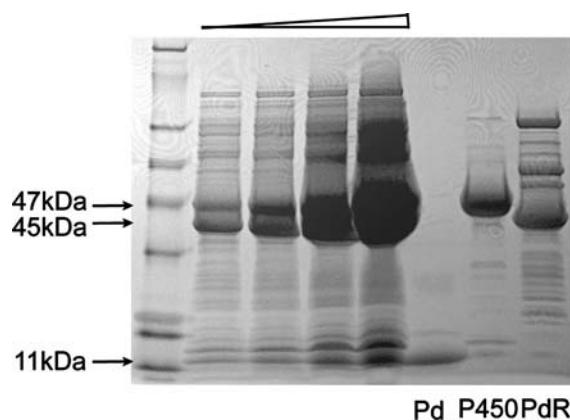


Fig. 2 SDS-PAGE/coomassie staining of soluble fraction. A molecular weight ladder is shown on the left, followed by four lanes with increasing concentrations of the expressed proteins, followed by lanes—as marked—for recombinant Pd, P450_{cam}, and PdR

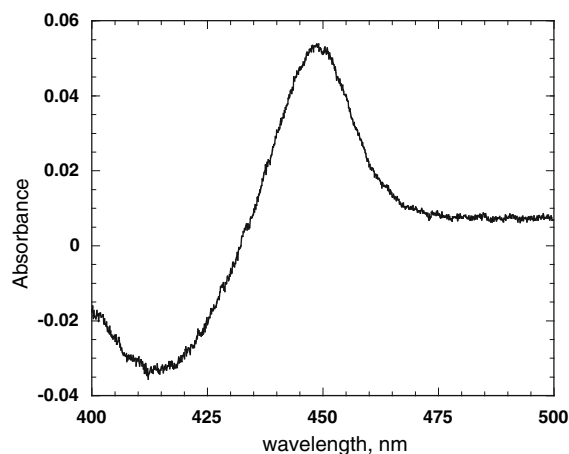


Fig. 3 Fe²⁺ vs. Fe²⁺–CO difference spectrum of a CYP101: Pd: PdR tricistronic expression culture

spectra were measured with concentrated whole cells (Fig. 3). The expression level was more than 500 nmol P450 holoenzyme per 1 l culture. The reductase activity of PdR in the tricistronic system was verified by the reduction of nitroblue tetrazolium. A clear reduction activity in *E. coli* whole cells expressing three cistronic operons was observed but no color change in the control cells including pCW'(Ori⁺) vector (Fig. 4). The calculated reductase activity of PdR was about 30 nmol NBT reduced/min/ml whole cell culture. These results confirm the clear expression of functional PdR in the constructed system.

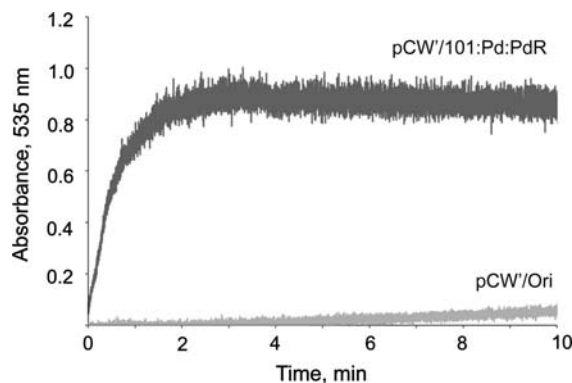


Fig. 4 Activity measurement of PdR in CYP101: Pd: PdR tricistronic expression system. After 1 mM NADH was added to the sample cuvette, the absorbance at 533 nm was monitored until there was no further increase

Camphor hydroxylation by *E. coli* whole cells expressing tricistronic operons

The camphor hydroxylation reaction products were analyzed by GC-MS after extraction with methylene dichloride. The 5-*exo*-hydroxycamphor was produced and identified by its molecular mass (m/z 168) (Supplementary Fig. 1). The 5-*exo*-hydroxycamphor was also produced in the reaction with the soluble fraction of the *E. coli* expression. These results indicate that the three proteins expressed in *E. coli* cells constitute a self-sufficient catalytic system.

L-Limonene hydroxylation by *E. coli* whole cells expressing tricistronic operons

The 7-hydroxylation of L-limonene by the tricistronic system was investigated for its bioengineering potential for the production of an anticancer compound (Reddy et al. 1997). The whole cell enzymatic reaction was performed as done for camphor hydroxylation. The production of (–)-perillyl alcohol was confirmed by cochromatography and mass spectrometric comparison of the metabolite with the authentic compound (Supplementary Fig. 2). No other metabolite was observed in the reaction. This result indicates that the 7-position is the major site of hydroxylation of L-limonene by P450_{cam} and suggests that this system may be applicable to the large scale production of this metabolite.

Discussion

In this study, we used a tricistronic system that produces bacterial P450 and two auxiliary proteins from a single mRNA. The coding region for the three proteins was connected by the T7 gene leader including a Shine-Delgarno ribosomal binding sequence. The polycistronic expression approach has advantages including accommodation of the natural environment for the reconstitution of three proteins in prokaryotes and no need to purify the proteins for enzyme reactions (Parikh et al. 1997). Previous attempts to construct a self-sufficient *E. coli* catalytic system using a fusion protein showed that the order of the three proteins is important for optimal enzymatic efficiency regardless of the length of the linker peptides (Sibbesen et al. 1996).

One concern regarding biotransformation in living cells is transport of the substrate into the cell and the product out of the cell, and the availability of an energy source. Many P450 substrates are sufficiently hydrophobic to penetrate the polysaccharide *E. coli* cell wall. Camphor and limonene tested in this study successfully accessed the cytosolic compartment in which the three recombinant proteins were expressed. The P450_{cam} reaction requires a coupled and stepwise supply of electrons from NADH (Paine et al. 2004). To circumvent the possibility of a limited NADH supply in the living cells, glucose was added as an energy source for the whole cell enzymatic reaction, as was previously shown with human CYP1A2 (Kim and Guengerich 2004).

Limonene, a monocyclic monoterpene, is present in orange peel and other plants and has chemopreventive activities. The production of (–)-perillyl alcohol from limonene is of interest because of the limited availability of (–)-perillyl alcohol in nature and its proven anticarcinogenic properties in the treatment of breast, pancreatic, and colorectal cancer (van Beilen et al. 2005). Human CYP2C19 was able to oxidize L-limonene to perillyl alcohol (Miyazawa et al. 2002). Recently, the production of perillyl alcohol from limonene using an alkane hydroxylase from *P. putida* was reported (van Beilen et al. 2005). Although P450_{cam} has traditionally been viewed as a camphor-specific enzyme, it metabolizes a diversity of compounds including limonene, as shown in this study, in a regio- and stereospecific manner. Therefore, the application of the tricistronic system in living cells for industrial production of (–)-perillyl alcohol in large-scale bioreactors is of potential interest.

Mutagenesis studies can provide information on the structure-function relationships of P450 proteins. However, it requires a high-throughput assay system including the complete reconstitution of P450, Pd, and PdR. Therefore, the development of this system presents the prospect of its use in mutagenesis studies of structure-function relationships, as well as its application in industrial bioreactions for the production of useful chemicals. Numerous orphan P450s are expressed in intriguing bacteria such as *Mycobacterium tuberculosis* and *Streptomyces coelicolor* (Lamb et al. 2006). However, it has been a challenge to establish a strategy for finding the substrates and metabolites of orphan P450s. Pd and PdR are known

to be able to transfer electrons to many bacterial P450 enzymes (Paine et al. 2004). Tricistronic systems cloned with the orphan bacterial P450s could provide an alternative strategy to make the initial substrate screening in living organism possible.

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