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Functional expression system for cytochrome P450 genes using the reductase domain of self-sufficient P450RhF from *Rhodococcus* sp. NCIMB 9784

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Abstract Cytochrome P450RhF from *Rhodococcus* sp. NCIMB 9784 is a self-sufficient P450 monooxygenase. We report here a simple system for the functional expression of various P450 genes using the reductase domain of this P450RhF, which comprises flavin mononucleotide- and nicotinamide adenine dinucleotide phosphate binding motifs and a [2Fe2S] ferredoxin-like center. Vector pRED was constructed, which carried the T7 promoter, cloning sites for a P450, a linker sequence, and the P450RhF reductase domain, in this order. The known P450 genes, encoding P450cam from *Pseudomonas putida* (CYP101A) and P450bzo from an environmental metagenome library (CYP203A), were expressed on vector pRED as soluble fusion enzymes with their natural spectral features in *Escherichia coli*. These *E. coli* cells expressing the P450cam and P450bzo genes could convert (+)-camphor and 4-hydroxybenzoate into 5-*exo*-hydroxycamphor and protocatechuate (3,4-dihydroxybenzoate), respectively (the expected products). Using this system, we also succeeded in directly identifying the function of P450 CYP153A as alkane 1-monooxygenase for the first time, i.e., *E. coli* cells expressing a P450 CYP153A gene named P450balk, which was isolated form *Alcanivorax borkumensis* SK2, converted octane into 1-octanol with high efficiency (800 mg/l). The system presented here may be applicable to the functional identification of a wide variety of bacterial cytochromes P450.

Introduction

Cytochromes P450 are a superfamily of heme-containing enzymes which are present in a wide range of organisms from bacteria to higher plants and mammals and catalyze monooxygenation reactions in a diversity of organic low-molecular-weight compounds. Their wide variety in *regio*- and *stereo*-specific reactions is likely to enable the production of industrially useful compounds that are difficult or impractical to synthesize by chemical methods and the decomposition of environmental pollutants that are tough to degrade (Kellner et al. 1997; Urlacher et al. 2004). The cytochromes P450 must be associated with electron donor partner protein(s) to transfer two electrons from nicotinamide adenine dinucleotide phosphate [NAD(P)H] to the heme domain of a P450 (Takemori et al. 1993). P450s generally fall into two broad classes depending on the nature of the redox partner(s) (Nebers and Gonzalez 1987). Class I cytochrome P450 monooxygenases, consisting of three components, contain two redox partner proteins, i.e., a flavin adenine dinucleotide (FAD)-including ferredoxin reductase and a ferredoxin that is a small iron–sulfur protein. Class I P450s are found in bacteria and the membranes of mitochondria. Class II cytochrome P450 monooxygenases, which comprise two components and are present in the liver microsomes of mammalian cells, contain an FAD and flavin mononucleotide (FMN)-including NADPH-dependent P450 reductase.

An exception is known that does not need the above-mentioned redox partner protein(s). The most well-known example is P450BM3, a fatty acid ω-2 hydroxylase, which has been found in *Bacillus megaterium* (Matson et al. 1977; Munro et al. 2000). This P450 monooxygenase is a self-sufficient single-polypeptide enzyme comprising the heme domain of P450 and a reductase domain, including the binding sites for FAD and FMN (Narhi and Fulco 1986). Thus, P450BM3 can be recognized as a special fused form of the class II cytochromes P450. P450BM3 or its evolved mutant has been studied to biotransform not only fatty acids but also aromatic hydrocarbons since it was functionally stable due to the fusion form (Li et al. 2001). New members belonging to the group of P450BM3 have also been found in *Bacillus subtilis* (Budde et al. 2004) and in such fungi as *Fusarium oxysporum* (Nakayama et al. 1996). The unique P450 monooxygenase, P450RhF, consisting of a self-sufficient single polypeptide, has recently

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been discovered in *Rhodococcus* sp. NCIMB 9784 (Roberts et al. 2002, 2003). P450RhF comprises the heme domain of the P450 and a C-terminal reductase portion containing FMN- and NAD(P)H-binding sites and a [2Fe2S] ferredoxin-like center. This reductase domain of P450Rhf is markedly different from that of P450BM3, which lacks a ferredoxin-like component. P450RhF is more analogous to a fused version of class I P450s, although in this case, the electrons are presumably transferred through FMN rather than FAD (Roberts et al. 2002).

The progress of genome analysis in this last decade has accelerated the discovery of many bacterial P450 genes belonging to class I cytochromes P450. For instance, it is known that 18 and 20 P450 genes exist in *Streptomyces coelicolor* (Bentley et al. 2002) and *Mycobacterium tuberculosis* (Cole et al. 1998), respectively. On the other hand, redox partner protein(s) corresponding to each P450 have hardly been detected in many cases; e.g., only 18 cytochromes P450 form a gene cluster, including ferredoxin, among a total of 174 P450s found in 45 *Streptomyces* species (Parajuli et al. 2004).

Redox partner proteins such as a ferredoxin reductase and ferredoxin derived from another organism have occasionally been used to successfully analyze the catalytic function of a P450. Several cytochromes P450 derived from *Streptomyces* sp. or other bacteria have been functionally identified by cooperating with putidaredoxin reductase and putidaredoxin, which are derived from *Pseudomonas putida* (Grogan et al. 2002; Uchiyama et al. 2005). P450EP1A from *Corynebacterium* sp. was able to degrade 2-ethoxyphenol by an in vitro assay composed of spinach ferredoxin-$NADP^+$ oxidoreductase (Kawahara et al. 1999). However, analyzing the catalytic function of a P450 by utilizing one or more foreign electronic donor proteins has been unsuccessful in many cases (Kellner et al. 1997), probably due to the low efficiency of electron transfer from the donor protein(s) to the P450. The construction of an efficient system for functionally expressing a variety of P450s belonging to class I P450s is therefore desirable for research into the biochemistry of P450s and their industrial use. We report here a simple system for the functional expression of various class I P450s derived from bacteria by using the reductase domain of self-sufficient P450RhF from *Rhodococcus* sp. NCIMB 9784. We also show the direct evidence that a P450 belonging to the CYP153A family catalyzes the reaction from *n*-alkane to 1-alkanol using this system.

Materials and methods

Bacterial strains

Escherichia coli TOP10F′ for the cloning experiments was purchased from Invitrogen, and *E. coli* BL21 (DE3) as an expression host was from Novagen. *Rhodococcus* sp. strain NCIMB 9784 was obtained from the National Collections of Industrial, Food, and Marine Bacteria, and *P. putida*ATCC17453 was from the Japan Collection of Microorganisms. *Alcanivorax borkumensis* SK2 (DSM 11573^T) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

Recombinant DNA techniques

The restriction enzymes and DNA ligation kit were purchased from New England BioLabs and Toyobo, respectively. DNA manipulation was conducted by the standard methods (Sambrook and Russell 2001) or as instructed by the appropriate kit supplier. Plasmid DNA was prepared with a Miniprep purification kit (Qiagen), and polymerase chain reaction (PCR) was carried out with an automated thermal cycler (Techne) using kinetic outlier detection (KOD) plus DNA polymerase (Toyobo). Total DNA was extracted from *Rhodococcus* sp. strain NCIMB 9784, *P. putida* strain ATCC17453, and *A. borkumensis* SK2 as previously described (Teramoto et al. 2003).

Construction of the expression vector

The expression vector for translating a fusion protein with the P450RhF reductase domain at the C-terminus was constructed by amplifying the DNA fragment encoding a P450RhF linker sequence [amino acid residue no. 445–460 in DNA Data Base of Japan (DDBJ)/GenBank accession no. AAM67416] and a reductase domain (amino acid residue no. 461–773) by PCR with primer sets 1 and 2 (Table 1), using genomic DNA from *Rhodococcus* sp. NCIMB 9784. Primer 1 contained an *Eco*RI restriction site upstream of the linker sequences of P450RhF, and primer 2 contained a *Sac*I site downstream of the 3′-terminal of the P450RhF gene. Nucleotides in these primers differing from the original genomic DNA sequence are underlined, these being introduced in consideration of the codon usage of *E. coli*. The PCR product was purified and digested with *Eco*RI and *Sac*I, and then inserted into the *Eco*RI–*Sac*I site of *E. coli* vector pET21a (Novagen) to generate vector pRED as shown in Fig. 1.

Construction of plasmids to produce P450s fused to the P450RhF reductase domain

The P450cam and P450balk genes were amplified by PCR from total DNA from *P. putida* ATCC17453 and *A. borkumensis* SK2, respectively. The P450bzo gene was amplified from Bzo71-8 of an environmental metagenome library (Uchiyama et al. 2005). The primers used for this amplification are listed in Table 1. Primers 8 and 9 for the P450bzo gene were used to introduce the mutation (shown in bold type) so as to replace the *Eco*RI site originally contained inside this gene without changing the amino acid. The N-terminal fragment amplified with primers 6 and 9 and the C-terminal fragment amplified with primers 7 and 8 were mixed, and then, the P450bzo mutant gene without the *Eco*RI restriction site was reassembled to full length with primers 6 and 7. The three amplified P450

Table 1 Oligonucleotides used in this study

Target clone	Primer	Sequence	Restriction enzyme
P450RhF linker and reductase domain	1	5′-GG*GAATTC*GTGCTGCACCGCCATCAACCG-3′	*Eco*RI
	2	5′-GG*GAGCTC*TCAGAGGCGCAGGGCCAGGCG-3′	*Sac*I
P450cam	3	5′-GACTAGT*CATATG*ACGACTGAAACCATACA-3′	*Nde*I
	4	5′-GG*GAATTC*TACCGCTTTGGTAGTCGCCGG-3′	*Eco*RI
	5	5′-GG*GAATTC*TAATACCGCTTTGGTAGTCGCCGGA-3′	*Eco*RI
P450bzo	6	5′-CGATATA*CATATG*TTCAGTTTTGACCCCTAT-3′	*Nde*I
	7	5′-GG*GAATTC*CGAAACGGCCAATTCCAG-3′	*Eco*RI
	8	5′-GGATTGCGTTTGACGAATT**T**CACAAA-3′	
	9	5′-GGGCTCACTTTGTG**A**AATTCGTCAAA-3′	
	10	5′-GC*GGATCC*TTACGAAACGGCCAATTCCAGCA-3′	*Bam*HI
P450balk	11	5′-CACTAGT*CATATG*TCAACGAGTTCAAGTACA-3′	*Nde*I
	12	5′-CAGCAC*CAATTG*TTTTTTAGCCGTCAACTT-3′	*Mfe*I
	13	5′-CAGCAC*CAATTG*TTATTTTTTAGCCGTCAA-3′	*Mfe*I

Italics show restriction sites

genes had the *Nde*I site at their 5′-ends and the *Eco*RI or *Mfe*I site at their 3′-ends. The fused P450 in pRED was constructed by removing the stop codon of each P450 gene (see primers 4, 7, and 12 in Table 1). The DNA fragment carrying the P450cam, P450bzo, and P450balk genes was inserted into the *Nde*I–*Eco*RI sites of pRED (Fig. 1) to yield plasmids named pCam-Red, pBzo-Red, and pBalk-Red, respectively. The sequences of the constructed fusion genes were confirmed to have no frame shift or mutation. As a comparative control, each gene encoding P450cam, P450bzo, and P450balk was amplified with primers 3 and 5, 6 and 10, and 11 and 13, respectively, and then cloned into the pET21a vector to generate control constructs pCam, pBzo, and pBalk.

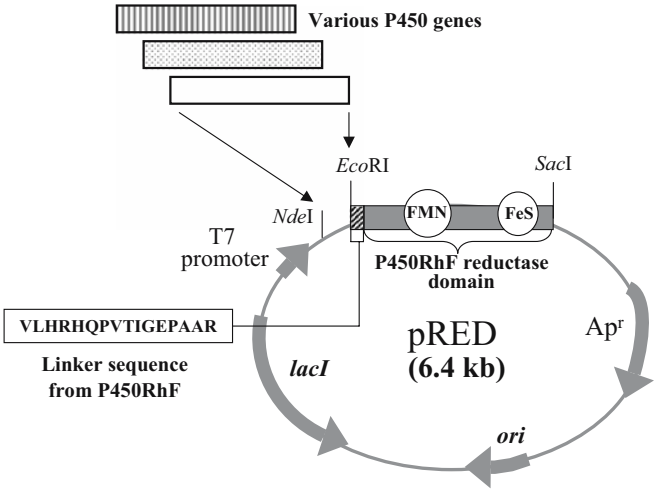

Fig. 1 Structure of vector pRED for functionally expressing a variety of class I P450s. The DNA fragment encoding the linker sequences and the reductase domain of P450RhF amplified by PCR was inserted into pET21a to generate expression vector pRED. Various class I P450 genes without their stop codon could be inserted into the *Nde*I–*Eco*RI site to produce hybrid P450s

Expression of respective P450s fused to the P450RhF reductase domain in *E. coli*

Individual plasmids were introduced in *E. coli* BL21 (DE3). Cultures of recombinants were grown in 40 ml of an M9 medium (Sambrook and Russell 2001) containing 0.4% glucose, 0.01% $FeCl_3$, and 150 μg/ml of ampicillin (Ap) for *E. coli* carrying pCam-Red or pCam, or a Luria–Bertani (LB) medium (Sambrook and Russell 2001) containing 150 μg/ml of Ap for *E. coli* (pBzo-Red or pBzo) and *E. coli* (pBalk-Red or pBalk) at 30°C with reciprocal shaking. After the absorbance at optical density (OD) 600 nm had reached 0.8, 0.4 mM of isopropylthio-β-D-galactoside (IPTG) and 0.5 mM of 5-aminolevulinic acid hydrochloride (ALA) were added, and then, cultivation was continued with reciprocal shaking for 16 h at 25°C [or 20°C for *E. coli* (pBalk-Red or pBalk)]. The cells were collected, suspended in 5 ml of a 50-mM phosphate buffer (pH 7.0), and disrupted by sonication. After centrifuging, the soluble fractions were filtered and then subjected to an SDS-PAGE analysis or spectrophotometric analysis.

Bioconversion of camphor by the *E. coli* cells harboring pCam-Red

Escherichia coli BL21 (DE3) carrying plasmid pCam-Red or pCam was grown on 50 ml of an M9 medium containing 0.4% glucose, 0.01% $FeCl_3$, and 150 μg/ml of Ap at 30°C with reciprocal shaking. The BL21 (DE3) strain containing the pET21a vector without an insert was also cultured as a negative control. After the absorbance at OD 600 nm had reached 0.7–0.8, 0.4 mM of IPTG and 0.5 mM of ALA were both added, and cultivation was continued for 16 h at 25°C with reciprocal shaking. The cells were collected and then suspended in 5 ml of a 50-mM phosphate buffer (pH 7.0) containing 0.5% glycerol that was added for the stabilization of the enzyme, and 0.5 mM of (+)-camphor (Wako) as the substrate. The reaction was performed at

25°C while shaking, aliquots of the reaction mixture being collected after 0, 3, 6, and 24 h of incubation. To analyze the converted product and substrate, 150 μl of ethyl acetate and 10 μl of 3N HCl were added to 100 μl of each reaction mixture, before the organic phase was separated by centrifugation. The product (5′-*exo*-hydroxyl camphor) was detected by drying and trimethylsilylating the extracted organic phase in vacuo. The resulting samples were analyzed by gas chromatography–mass spectrometry (GC-MS).

Bioconversion of 4-hydroxybenzoate by *E. coli* cells harboring pBzo-Red

Escherichia coli BL21 (DE3) carrying plasmid pBzo-Red or pBzo was grown under the same conditions as those used for *E. coli* carrying pBalk-Red or pBalk. The cells were collected and then suspended in 10 ml of a 50-mM phosphate buffer (pH 7.0) containing 0.5% glycerol and 0.5 mM of 4-hydroxy benzoate (Tokyo Kasei Kogyo) as the substrate. Aliquots of the reaction mixture were collected after 0, 2, 4, and 6 h of incubation at 20°C while shaking. The organic phase of each sample extracted as mentioned above was dried in vacuo and trimethylsilylated before being analyzed by GC-MS.

Bioconversion of *n*-octane by *E. coli* cells harboring pBalk-Red

Escherichia coli BL21 (DE3) carrying plasmid pBalk-Red or pBalk was grown in 100 ml of an LB medium containing 150 μg/ml of Ap at 30°C with reciprocal shaking. After the absorbance at OD 600 nm had reached 0.8, 0.4 mM of IPTG and 0.5 mM of ALA were both added, and cultivation was continued for 16 h at 20°C with reciprocal shaking. The biotransformation of *n*-octane using the recombinant *E. coli* cells was basically carried out according to the method reported by Fujii et al. (2004). The cells were collected and suspended in 5 ml of a 50-mM phosphate buffer (pH 7.0) containing 0.5% glycerol and whose1.5 ml was each put in five 12-ml test tubes with a screw cap. *n*-Octane (500 μl, Wako) was added to the test tubes before being incubated at 20°C with shaking at 100 rpm. The test tubes were taken out one by one after 0, 2, 4, 8, and 24 h of incubation, and aliquots of the reaction mixture were collected. The converted product (1-octanol) in the octane phase was identified and quantified by a GC-MS analysis.

GC-MS of the converted products

The resulting samples were analyzed by GC-MS (Shimadzu QP5050A instrument) with a DB-5 column (30-m length; 0.25-mm diameter; J&W Scientific) to identify the converted products and substrates. The injection and detection temperatures were both 250°C. 1-Octanol was subjected to an initial temperature of 80°C for 3 min, with the temperature then being raised to 105°C at a rate of 5°C/min and then to 240°C at a rate of 20°C/min. The other analyses were conducted with the column temperature initially kept at 60°C for 6 min before being increased to 260°C at a rate of 10°C/min. The substrate and products were quantified by a selective ion monitoring (SIM) program generating standard curves of each compound.

Results

Expression of the genes encoding P450s fused to the RhF reductase domain in *E. coli*

Cell-free extracts composed of soluble proteins were prepared from *E. coli* BL21 (DE3) expressing plasmids pCam-Red, pBalk-Red and pBzo-Red and subjected to an SDS-PAGE analysis (Fig. 2). This analysis showed that the three hybrid P450s fused to the RhF reductase domain of *Rhodobacter* sp. NCIMB 9784 (fusion P450s), P450cam-Red, P450bzo-Red, and P450balk-Red had been correctly produced in the soluble fractions of the *E. coli* cells (Fig. 2). The theoretical molecular masses of P450cam-Red, P450bzo-Red, and P450balk-Red were 82.3, 79.3, and 89.1 kDa, respectively, these values being deduced by taking the P450RhF reductase domain as 35,591 Da. The molecular masses of the detected bands, shown in Fig. 2 by arrows, are consistent with the theoretical values.

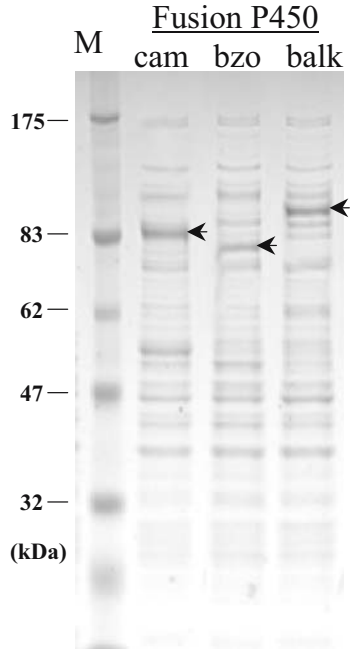

Fig. 2 Synthesis of the P450s fused to the reductase domain of P450RhF in *Escherichia coli*. The results of an SDS-PAGE analysis (7.5–15%) with Coomassie Brilliant Blue staining are shown. *Lanes*: cam, bzo, and balk show the soluble fractions from *E. coli* carrying pCam-Red, pBzo-Red, and pBalk-Red, respectively. *Arrows* indicate the position of the recombinant proteins. Molecular mass markers are shown in *lane M*

Spectrophotometric analysis of the fusion P450s produced in *E. coli*

The spectral characteristics of the P450cam-Red fusion protein in a cell-free extract were analyzed. The absorption spectra (350–500 nm) of P450cam-Red (Fig. 3a) in its oxidized form (solid line), reduced carbon monoxide (CO)-binding state (dotted line), and substrate-binding state (dashed line) reveal the same features as those of typical bacterial P450s (Lewis 1996). The CO differential spectra resulting from the addition of CO to the hydrosulfite-reduced P450 showed the heme Soret absorption maxima at 450 nm (inset). When P450cam-Red was mixed with camphor, the induced spectral change was from a maximum peak at 418 nm (solid line) to around 390 nm (dashed line). Indeed, the original P450cam produced by control plasmid pCam exhibited the same spectra pattern as that of P450cam-Red (Fig. 3b). The other fusion P450s also showed movement of the maximum peak from 420 to 450 nm in the reduced CO-binding spectra and that of the maximum peak from 420 to 390 nm in the substrate-binding state (data not shown).

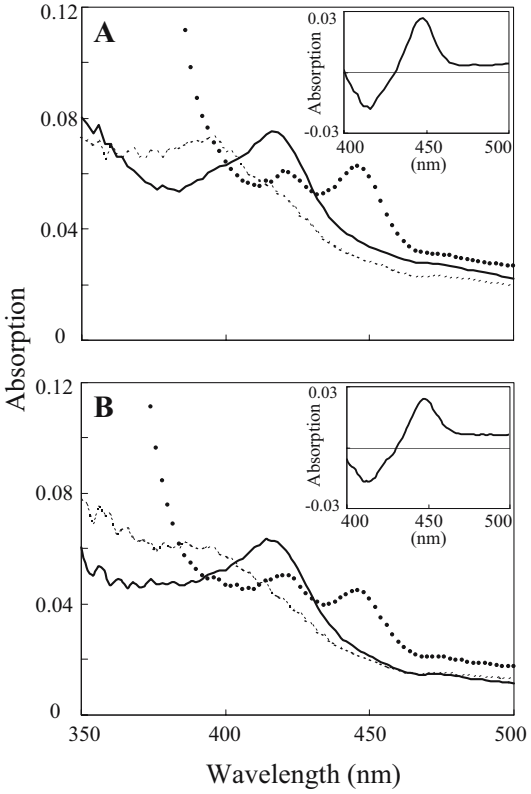

Fig. 3 Spectral features of the fusion P450cam (P450cam-Red) (**a**) and control P450cam not containing a reductase domain (**b**) in the cell-free extract. Spectra are shown for the oxidized (*solid line*), sodium-hydrosulfite-reduced and then carbon-monoxide-bound (*dotted line*), and substrate-binding (*dashed line*) forms of the enzymes. The CO difference spectrum for each enzyme is shown in the *inset*

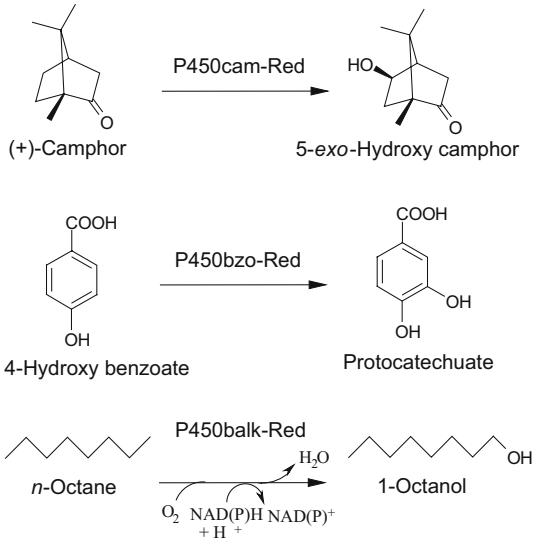

Fig. 4 Biotransformation of the respective substrates by recombinant *E. coli* cells synthesizing the fusion P450s. Bioconversion from *n*-octane to 1-octanol by the recombinant *E. coli* cells expressing the P450 gene is demonstrated in this study for the first time

Bioconversion of camphor by *E. coli* BL21 (DE3) synthesizing P450cam fused to the RhF reductase domain (P450cam-Red)

It is well known that P450cam from *P. putida* catalyzes the hydroxylation of (+)-camphor to produce 5′-*exo*-hydroxyl camphor (Fig. 4) when the P450 cooperates with putidaredoxin reductase and putidaredoxin derived from the same bacterial species (Koga et al. 1985). Whole-cell bioconversion experiments were performed using *E. coli* (pCam-Red) synthesizing P450cam-Red and the control strain *E. coli* (pCam) synthesizing P450cam not including the reductase domain, the resting cells being incubated with 0.5 mM of (+)-camphor at 25°C (Fig. 5a). After a 24-h reaction, *E. coli* (pCam-Red) achieved 100% conversion of camphor to produce 5′-*exo*-hydroxyl camphor (Fig. 5a). On the other hand, *E. coli* (pCam) showed low conversion activity, in which there was gradual loss of the substrate and gradual formation of the product. It was also confirmed that there was no activity in the negative control *E. coli* (pET21a).

Bioconversion of 4-hydroxybenzoate by *E. coli* BL21 (DE3) synthesizing P450bzo fused to the RhF reductase domain (P450bzo-Red)

P450bzo was a new CYP203A member found in Bro71-8 from the environmental metagenome library, this being recently constructed and based on benzoate induction, and was found to catalyze the 3-hydroxylation reaction of 4-hydroxyl benzoate when the P450 cooperated with putidaredoxin reductase and putidaredoxin derived from *P. putida* in vitro (Uchiyama et al. 2005). We performed bioconversion experiments, using 4-hydroxybenzoate as

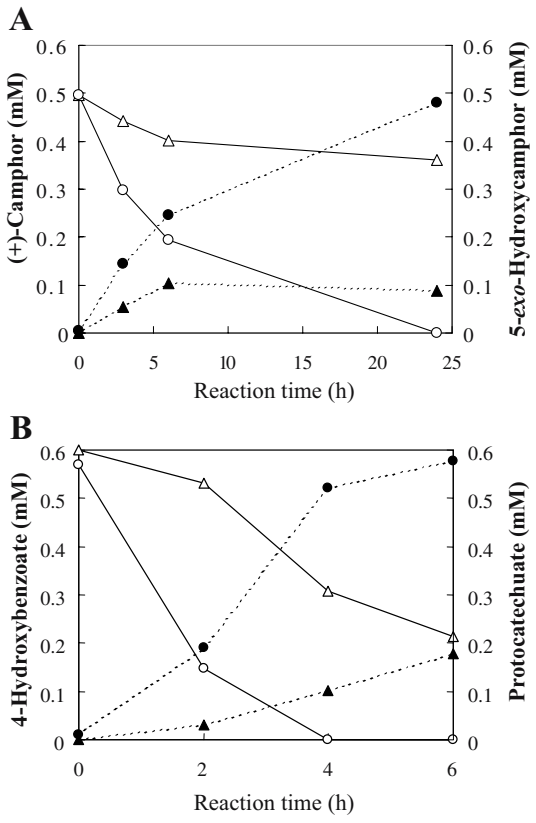

Fig. 5 **a** Biotransformation of camphor by *E. coli* (pCam-Red) (*circles*) and *E. coli* (pCam) control (*triangles*). **b** Biotransformation of 4-hydroxybenzoate by *E. coli* (pBzo-Red) (*circles*) and *E. coli* (pBzo) control (*triangles*). The substrates (camphor and 4-hydroxybenzoate) and products (5-*exo*-hydroxycamphor and protocatechuate) are shown by *open* and *filled symbols*, respectively. Three independent experiments were done, and the average was shown

the substrate, to produce protocatechuate (3,4-dihydroxybenzoate) by *E. coli* (pBzo-Red) and the control recombinant *E. coli* (pBzo) not including the reductase domain (Fig. 4). Figure 5b shows the amounts of the substrate and product in each sample after 2, 4, and 6 h of the reaction. The strain synthesizing P450bzo-Red could completely convert the substrate after 4 h of the reaction, while half of the substrate remained with the control strain after the same reaction time.

Bioconversion of *n*-octane by *E. coli* BL21 (DE3) synthesizing P450balk fused to the RhF reductase domain (P450balk-Red)

Cytochromes P450 belonging to the CYP153A family have been thought to catalyze oxidation of *n*-alkane to produce 1-alkanol, although no direct evidence has been reported so far (Maier et al. 2001). We isolated a P450 gene that belongs to this family, named P450balk, form *A. borkumensis* SK2, and cloned into vector pRED to generate pBalk-Red. The nucleotide sequence of P450balk is available from databases (DDBJ accession no. AY505118 and http://drnelson.utmem.edu/biblioE.html). The biocon-

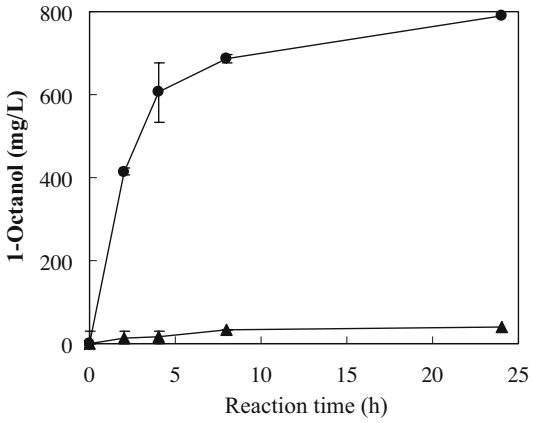

Fig. 6 Biotransformation of *n*-octane by *E. coli* (pBalk-Red) (*circles*) and *E. coli* (pBalk) control (*triangles*). The concentration of the product (1-octanol) in the octane phase was measured after 4, 8, and 24 h of the reaction. Three independent experiments were done, and the average was shown

version of *n*-octane was performed using *E. coli* cells carrying plasmid pBalk-Red. As the reaction proceeded, the formation of 1-octanol was detected as the result of hydroxylation of *n*-octane (Figs. 4 and 6). After 24 h of the reaction with *E. coli* (pBalk-Red), about 800 mg/l of 1-octanol had accumulated in the octane phase, whereas the control strain *E. coli* (pBalk) not including the reductase domain produced 40 mg/l of 1-octanol, this being only 5% of the product with the P450balk-Red fusion protein. No product other than 1-octanol was detected, and the negative control *E. coli* (pET21a) did not produce 1-octanol at all.

Discussion

Cytochrome P450RhF of *Rhodococcus* sp. NCIMB 9784 that has recently been reported by Roberts et al. (2002, 2003) is the only self-sufficient P450 monooxygenase analogous to a fused version of class I cytochromes P450 rather than to class II P450s. We constructed in this present study a simple and efficient system for functionally expressing a variety of class I P450 genes derived from bacteria by using the reductase domain of this P450RhF, which comprises FMN- and NAD(P)H-binding motifs and a [2Fe2S] ferredoxin-like center, the linker sequence comprising the 16 amino acids derived from P450RhF being preceded (Fig. 1). The expression vector, pRED, which carries the T7 promoter, the cloning sites for a P450, the linker sequence, and the P450RhF reductase domain, in that order, was constructed using *E. coli* vector pET21a (Fig. 1). The use of this expression vector enabled us to demonstrate the functional expression of three bacterial genes encoding class I cytochromes P450, these being P450cam from *P. putida* (CYP101A), P450bzo obtained from an environmental metagenome library (CYP203A), and P450balk from *A. borkumensis* SK2 (CYP153A). Each of these three hybrid P450s incorporating the reductase domain at the C-terminus was produced as soluble and functional enzymes in the recombinant *E. coli* cells (Figs. 2

and 3), and their catalytic activities were found to be stable and at a sufficiently high level for bioconversion of the substrates to the products (Figs. 5 and 6). The control *E. coli* strains containing only the P450 proteins could have the weak ability to biotransform the substrates. It had been reported that flavodoxin and NADPH-flavodoxin reductase, the endogenous proteins in *E. coli*, acted as electron donors to several foreign P450s (Blasco et al. 2004; Jenkins and Waterman 1994). Thus, it is likely that the flavodoxin and NADPH-flavodoxin reductase of *E. coli* also work for the three P450s used in this study. Specifically, P450bzo seems to have good affinity to the *E. coli* redox proteins as the electron donor.

The sequences of several P450 CYP153A genes are shown in a database (http://drnelson.utmem.edu/biblioE.html). These P450s, which have been isolated form alkane-utilizing bacteria such as *Acinetobacter* sp. EB104 and *A. borkumenes*, have been thought to mediate the terminal hydroxylation reactions of alkanes (Maier et al. 2001). However, it was difficult to demonstrate the formation of 1-alkanols from the substrates in vitro even in the presence of putidaredoxin and putidaredoxin reductase (our unpublished results). P450s belonging to CYP153A may be unstable proteins. By using the functional expression system developed in this work, we succeeded in directly identifying the catalytic function of the P450 CYP153A as alkane 1-monooxygenase for the first time, i.e., *E. coli* expressing the P450 CYP153A gene isolated form *A. borkumensis* SK2, named P450balk, was found to convert octane into 1-octanol. The P450balk protein fused to the P450RhF reductase domain via the appropriate linker sequence seems to be very stable in *E. coli* cells. It is, however, awaiting further consideration whether the fusion protein extracted from the cells is sable or not, compared with the P450balk protein (not fused), and whether P450balk prefers the P450RhF reductase domain to a putidaredoxin and putidaredoxin reductase system as the electron donor from NAD(P)H to the P450.

The recombinant *E. coli* cells carrying plasmid pBalk-Red produced a great amount of 1-octanol (800 mg/l) after a 1-day incubation. It is well known that nonheme alkane hydroxylases (AlkB) catalyze such monooxygenation reactions of alkanes to synthesize 1-alkanol (Eggink et al. 1987; Whyte et al. 2002; Hara et al. 2004). It was recently reported that *E. coli* expressing the *alkB2* gene isolated from *Gordonia* sp. TF6 could biotransform octane to produce 74 mg/l of 1-octanol when expressed along with the rubredoxin and rubredoxin reductase genes derived from the same strain (Fujii et al. 2004). Its production level had reached the maximum among those achieved by bioconversion experiments of alkanes with recombinant *E. coli* expressing *alkB*. Our system using the P450balk gene has achieved ten times the production level of this alkane hydroxylase (AlkB2) system. Considering that both bioconversion experiments were carried out under almost the same condition, the P450 system seems to be superior to the nonheme AlkB system in the case of using *E. coli* as the productive host.

The coexpression of the genes coding for a P450 and its redox partner protein(s) has occasionally biotransformed substrates with the improved conversion level, using a bicistronic system on one plasmid or a colocalization system on distinct plasmids (Shet et al. 1997). On the other hand, a system for utilizing a P450 fused to its redox partner(s) can be expected to provide us with simpler and more convenient methods for the functional expression of the P450 in an appropriate host, since only the expression of a single gene is needed, and for enzyme purification (e.g., using ADP-Sepharose affinity column), avoiding the need for the reconstitution of the purified redox partner proteins for in vitro study. In the present study, the P450RhF reductase domain was selected as the electron donor for fusing a cytochrome P450. It is because we expected that it was more reasonable to mimic the structure of P450RhF than to separate only the reductase domain portion of P450RhF as one additional protein for the coexpression system. The fusion system presented here may be applicable to the functional identification of a wide variety of class I cytochromes P450, many of whose catalytic functions have not been elucidated or demonstrated. The functional analysis of new P450 genes using this system, which were obtained from an environmental metagenome library will soon be published elsewhere.

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References

Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3 (2). Nature 417:141–147

Blasco F, Kauffmann I, Schmid RD (2004) CYP175A1 from *Thermus thermophilus* HB27, the first beta-carotene hydroxylase of the P450 superfamily. Appl Microbiol Biotechnol 64: 671–674

Budde M, Maurer SC, Schmid RD, Urlacher VB (2004) Cloning, expression and characterisation of CYP102A2, a self-sufficient P450 monooxygenase from *Bacillus subtilis*. Appl Microbiol Biotechnol 66:180–186

Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE III, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver S, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton S, Squares S, Squares R, Sulston JE, Taylor K, Whitehead S, Barrell BG (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393:537–544

Eggink G, Lageveen RG, Altenburg B, Witholt B (1987) Controlled and functional expression of the *Pseudomonas olevorans* alkane utilizing system in *Pseudomonas putida* and *Escherichia coli*. J Biol Chem 262:17712–17718

Fujii T, Narikawa T, Takeda K, Kato J (2004) Biotransformation of various alkanes using the *Escherichia coli* expressing an alkane hydroxylase system from *Gordonia* sp. TF6. Biosci Biotechnol Biochem 68:2171–2177

Grogan G, Roberts GA, Parsons S, Turner NJ, Flitsch SL (2002) P450(camr), a cytochrome P450 catalysing the stereospecific 6-endo-hydroxylation of (1 R)-(+)-camphor. Appl Microbiol Biotechnol 59:449–454

Hara A, Baik S-H, Syutubo K, Misawa N, Smits THM, van Beilen JB, Harayama S (2004) Cloning and functional analysis of *alkB* genes in *Alcanivorax borkumensis* SK2. Environ Microbiol 6:191–197

Jenkins CM, Waterman MR (1994) Flavodoxin and NADPH-flavodoxin reductase from *Escherichia coli* support bovine cytochrome P450c17 hydroxylase activities. J Biol Chem 269: 27401–27408

Kawahara N, Ikatsu H, Kawata H, Miyoshi S, Tomochika K, Sinoda S (1999) Purification and characterization of 2-ethoxyphenol-induced cytochrome P450 from *Corynebacterium* sp. strain EP1. Can J Microbiol 45:833–839

Kellner DG, Maves SA, Sligar SG (1997) Engineering cytochrome P450s for bioremediation. Curr Opin Biotechnol 3:274–278

Koga H, Rauchfuss B, Gunsalus IC (1985) P450cam gene cloning and expression in *Pseudomonas putida* and *Escherichia coli*. Biochem Biophys Res Commun 130:412–417

Lewis DF (1996) Cytochromes P450: structure, function and mechanism. Taylor & Francis, London

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

Maier T, Forster HH, Asperger O, Hahn U (2001) Molecular characterization of the 56-kDa CYP153 from *Acinetobacter* sp. EB104. Biochem Biophys Res Commun 286:652–658

Matson RS, Hare RS, Fulco AJ (1977) Characteristics of a cytochrome P-450-dependent fatty acid omega-2 hydroxylase from *Bacillus megaterium*. Biochim Biophys Acta 487:487–494

Munro AW, Noble MA, Ost TW, Green AJ, McLean KJ, Robledo L, Miles CS, Murdoch J, Chapman SK (2000) Flavocytochrome P450 BM3 substrate selectivity and electron transfer in a model cytochrome P450. Subcell Biochem 35:297–315

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

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

Nebers DW, Gonzalez FJ (1987) P450 genes: structure, evolution and regulation. Annu Rev Biochem 56:945–993

Parajuli N, Basnet DB, Chan LH, Sohng JK, Liou K (2004) Genome analyses of *Streptomyces peucetius* ATCC 27952 for the identification and comparison of cytochrome P450 complement with other *Streptomyces*. Arch Biochem Biophys 425:233–241

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

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. Biol Chem 278:48914–48920

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Shet MS, Fisher CW, Estabrook RW (1997) The function of recombinant cytochrome P450s in intact *Escherichia coli* cells: the 17 alpha-hydroxylation of progesterone and pregnenolone by P450c17. Arch Biochem Biophys 339:218–225

Takemori S, Yamazaki T, Ikushiro S (1993) Cytochrome P-450-linked electron transport system in monooxygenase reaction. In: Omura T, Ishimura Y, Fujii-Kuriyama Y (eds) Cytochrome P450, 2nd edn. VCH, New York

Teramoto M, Takaichi S, Inomata Y, Ikenaga H, Misawa N (2003) Structural and functional analysis of a lycopene β-monocyclase gene isolated from a unique marine bacterium that produces myxol. FEBS Lett 545:120–126

Uchiyama T, Abe T, Ikemura T, Watanabe K (2005) Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. Nat Biotechnol 23:88–93

Urlacher VB, Lutz-Wahl S, Schmid RD (2004) Microbial P450 enzymes in biotechnology. Appl Microbiol Biotechnol 64:317–325

Whyte LG, Smits THM, Labbe D, Witholt B, Geer CW, van Beilen JB (2002) Gene cloning and characterization of multiple alkane hydroxylase systems in *Rhodococcus* strains Q15 and NRRL B-16531. Appl Environ Microbiol 68:5933–5942