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Engineering Cytochrome P-450s

Chimeric Enzymes

Suja Sukumaran,[1] William Mark Atkins,[2] and Rishi Shanker*,[1]

[1]National Environmental Engineering Research Institute, Nehru Marg, Nagpur 440020, India, E-mail: rishi@nagpur.dot.net.in; and [2]Department of Medicinal Chemistry, University of Washington, Seattle, WA 98195, USA

Abstract

Cytochrome P-450 isozymes represent a critical component of nature's spectrum of detoxification catalysts that could be exploited for bioremediation. The ethanol-inducible human cytochrome P-450 2E1 serves as a model eukaryotic P-450 that complements the bacterial P-450 cam in dehalogenation and detoxification of environmental pollutants. We explored the construction of novel chimeric P-450s using cytochrome P-450 *camC* and *2E1* genes. For construction of chimera 1 (478 amino acids, 55.14 kDa), 145 amino acids from the N-terminus of P-450 2E1 protein (493 amino acids, 56.84 kDa) were replaced with 130 amino acids from the N-terminus of P-450 camC protein (415 amino acids, 46.66 kDa). In chimera 2 (525 amino acids, 60.24 kDa) the strategy involves replacement of 28 amino acids in the C-terminus of chimera 1 with 75 amino acids from the C-terminus of P-450 camC gene. Homology models of both the chimeric proteins were developed using SWISS-MODEL based on the known crystal structure of cytochrome P-450 camC, BM-3, 1DT6A, and 2C17A. The models indicated that the proposed heme-binding site was intact, which is inevitable for catalytic activity of cytochrome P-450s. The expression of chimera 1 and 2 genes in *Escherichia coli* DH5α was evident from light-pink cell pellets, protein band in sodium dodecyl sulfate polyacrylamide gel electrophoresis, and diagnostic carbon monoxide-difference spectra. Our studies show that strategies can be developed to exploit the natural diversity of the P-450 superfamily to generate chimeric biocatalysts that would provide new templates amenable to directed evolution.

Index Entries: Designer proteins; cytochrome P-450; P-450 101; chimeric P-450 2E1; homology modeling; camphor; detoxification; bacteria.

*Author to whom all correspondence and reprint requests should be addressed.
Present Address: Industrial Toxicology Research Center, PO Box 80, M. G. Marg, Lucknow-226001, India, E-mail: rishishanker1@rediffmail.com

Introduction

Natural microorganisms collectively exhibit remarkable evolutionary capabilities to adapt to a wide range of chemicals. However, natural evolution occurs at a relatively slow rate, particularly when the acquisition of multiple catalytic activities is necessary. Innovative approaches and strategies are needed to construct organisms that are known to actively survive with desired catabolic or detoxification potential in contaminated soils *(1)*. Recent molecular techniques have provided the tools for engineering microbes or enzymes to function as "designer biocatalysts," in which certain desirable traits from different organisms can be brought together to perform specific bioremediation *(2)*.

Cytochrome P-450s form a broad group of heme-containing monooxygenases that catalyze transformation of a wide range of substrates including xenobiotics. Therefore, cytochrome P-450 enzymes are of considerable environmental relevance *(3,4)*. This also makes cytochrome P-450s an interesting and a logical choice for protein engineering as well as construction of organisms that detoxify pollutants *(5)*. The best known and most extensively characterized of the bacterial enzymes is the cytochrome P-450 cam from camphor utilizing *Pseudomonas putida* G786. The P-450 cam monooxygenase system catalyzes hydroxylation of camphor to hydroxycamphor. The oxygenation event is catalyzed by a soluble three-protein system (4.47-kb *cam CAB* genes) linking the pyridine nucleotide dehydrogenation by flavin adenine dinucleotide flavoprotein, putidaredoxin reductase (*camA*) to the iron-sulfur protein, and putidaredoxin (*camB*)-mediated electron transport to cytochrome P-450 cam (*camC*). P-450 cam has been demonstrated to catalyze both oxidative and reductive reactions and can accommodate molecules such as camphor or pyrene to hexachloroethane at the active site *(6)*. Under anaerobic conditions, the cytochrome P-450 cam from *P. putida* G786 catalyzes dehalogenation by two-electron reduction and elimination *(7)*.

A eukaryotic P-450 of considerable relevance in xenobiotic metabolism is the ethanol-inducible cytochrome P-450 2E1. Like P-450 cam, the human P-450 2E1 mediates oxidative and reductive chemistry of a broad array of xenobiotics including *p*-nitrophenol, chloraxozone, *N*,*N*-dimethylnitrosamine, carbon tetrachloride, trichloroethylene, and halothane *(8)*. The attempts made for expression of human cytochrome P-450s 2E1 or 1A1 in *Escherichia coli* have proven invaluable in the characterization of these important enzymes *(9–12)*. Unfortunately, bacteria not only lack the endoplasmic reticulum (needed for binding of mammalian P-450s to the microsomal membrane) but also the requisite electron transfer partner, NADPH-dependent cytochrome P-450 reductase. Therefore, P-450s expressed in *E. coli* bind to inner bacterial membrane. Pritchard et al. *(13)* explored the heterologous expression of unmodified recombinant human P-450 2E1, 3A4, and 2A6 in *E. coli* by making NH_2-terminal translational fusions to bacterial leader sequences (*pel B* and *omp A*). They reported that

both leader constructs produced spectrally active, functional proteins. However, the membrane P-450 content was no higher than reported for P-450 2E1 in which the NH_2-terminal had been modified or removed *(9)* or when CYP 2E1 was coexpressed with NADPH-cytochrome P-450 reductase in *E. coli (14)*.

Interestingly, only a few observations are available on the activity of human cytochrome P-450 or the reductase-P450 fusion constructs in live bacterial cultures. The generation of catabolically active, heterologous monooxygenase systems in live, robust soil bacteria remains one of the challenges of contemporary environmental biotechnology. The feasibility of expression of selected bacterial and mammalian cytochrome P-450 monooxygenase(s) in an environmentally robust organism such as *Pseudomonas* for detoxification of a wide range of halogenated and aromatic compounds needs to be explored. Only then can the detoxification potential of multiple cytochrome P-450 enzymes be exploited by developing a coculture or consortium of bacteria expressing the isozymes. One major hurdle with this approach results from the difficulty in coexpression of mammalian P-450s and their required reductases and cytochrome-b_5 in soil bacteria. A generally applicable system would require a host bacterium that allows the expression of prokaryotic and eukaryotic P-450s and bypasses the requirement for "individualized" or additional electron transfer proteins. We have reported earlier the feasibility of a "universal" electron transfer mechanism that involved the construction of a strain of *P. putida* coexpressing the *Vibrio harveyi* luciferase *luxAB* and cytochrome P-450 *camC* genes in which the luciferase serves either as a bioluminescence catalyst for photoreduction or as an electron transfer partner for P-450 cam instead of the native electron transfer partner proteins, putidaredoxin and putidaredoxin reductase *(15,16)*. We have also constructed a *P. putida* coexpressing N-terminus-modified P-450 2E1 (21 amino acids deleted) and luciferase. The luciferase-dependent photoreduction of P-450 2E1 was also observed in *P. putida (17)*.

The construction and expression of eukaryotic P-450s in bacteria can be immensely useful in terms of detoxification capability. For example, the P-450 enzymes from higher organisms can catalyze oxygen incorporation in various haloalkanes and haloalkenes at rates much higher than bacterial enzymes *(3,5,8)*. However, creating enzymes with desired activity remains a challenging and formidable task since in most cases we can consider only amino acid residues that constitute the active site. It is beyond our present knowledge to predict *a priori* the effects of the mutations of remote residues on enzymatic activity through changes in the complex architecture of tertiary and/or quaternary structure. Construction of chimeric enzymes from two functionally related proteins sharing extensive sequence similarity is a possible first step in this direction *(18,19)*. This can provide invaluable information on the structure-function relationship of the parent proteins besides generating enzymes with improved catalytic properties *(20)*. A very useful strategy for protein engineering is assigning function to protein

motifs (by identifying potential fusion points for exchange of domains within enzyme superfamilies) for design of domain-swapped enzymes *(21,22)*. This strategy mimics evolutionary processes of domain swapping and can provide templates for rational redesign of enzymes by directed evolution *(23)*.

In the present study, we explored a possible strategy that can facilitate P-450 2E1 expression in both *E. coli* and *Pseudomonas* by fusion of the N- or both the N- and C-terminus of bacterial cytochrome P-450 camC gene to human cytochrome P-450 2E1 to generate chimeric enzymes.

Materials and Methods

Bacteria, Media, and Plasmid Vectors

The bacterial strains *E. coli* DH5α and *Pseudomonas* variants were grown in Luria broth or terrific broth (Difco, Detroit, MI). The broad host range plasmid vector pMMB 206 (9.311 kb; chloramphenicol as drug-resistance marker) was obtained from Dr. Mira Bagdasarian, University of Michigan, East Lansing *(24)*. The plasmid vectors' DNA was purified by Qiagen Q-500 columns (Qiagen) and low-melt agarose gel purification kits (Life-Technologies). For expression of cytochrome P-450 genes in *E. coli*, the pPROEX™ HTa (4.750 kb; ampicillin as drug-resistance marker) expression system was used (Life Technologies).

Primers, Polymerase Chain Reaction Amplifications, and Subcloning

The oligomers and polymerase chain reaction (PCR) conditions used are delineated in Table 1. The primers used were designed/modified by using Lasergene and Oligo5 softwares. The PCR oligomers were obtained from Integrated DNA Technologies or Life Science Technologies. The PCR amplifications were carried out using the Expand™ High Fidelity PCR System using the recommended concentrations of primers, dNTPs, $MgCl_2$, fidelity PCR enzyme mix, and template DNA with slight modification, whenever required (Boehringer-Mannheim). Alternatively, *Pfu* polymerase was used for long-template amplification (Promega, Madison, WI). Native cytochrome 2E1 gene in SK^- vector and total DNA from PpW-luxcam cells *(15)* were used as positive and negative PCR controls, respectively. The PCR amplifications were carried out on a Perkin-Elmer Thermal Cycler 9600 or an MJ Research DNA Engine PTC-200. Cohesive and blunt-end ligations were carried out using a Rapid DNA Ligation Kit according to the manufacturer's instructions (Boehringer-Mannheim). The transformation of competent cells of *E. coli* was carried out by electroporation using a Gibco-BRL Cell-Porator or chemical transformation protocol *(25)*. The colonies on the plates were screened by alkaline lysis miniplasmid preparations and restriction digestions. The clones were confirmed by PCR amplifications.

Table 1
Primers and PCR Thermal Cycling Conditions

Primers and PCR conditions	Reference
Cytochrome P-450 2E1	
2E1 P1: 5'-C GGG CTG CAG GAA TTC *ATG* GCT CGT CAA GTT CAT TCT TCT TGG AAT CTG CCC-3'	Modified from refs. *9* and *26*
2E1 LP2: 5'-AGG GCT GCA GGG TGT CCT CCA CAC ACT CAT GAG CGG-3'	
Cycles 1–35: denaturation for 75 s at 94°C; annealing for 35 s at 82°C; extension for 165 s at 72°C.	
Cycle 36: elongation for 420 s at 72°C.	
Product: 1461 bp	
Cytochrome P-450 cam C	
CE-1: 5'-GGC ACT TGA ATT CGT CAA GGC-3'	This study
CMCX 5'-ATTACCCTCGAGTCCCCCACCAA-3'	
Cycles 1–35: denaturation for 60 s at 94°C; annealing for 60 s at 59°C; extension for 165 s at 72°C.	
Product: 1481 bp	
CE-1: 5'-GGC ACT TGA ATT CGT CAA GGC-3'	This study
101-RP: 5'-GCC ACA CAT CCT CTT GGC TTC G-3'	
Cycles 1–35: denaturation for 60 s at 94°C; annealing for 90 s at 60°C; extension for 120 s at 72°C.	
Product: 822 bp	
Cytochrome P-450 cam C-2E1	
CE-1: 5'-GGC ACT TGA ATT CGT CAA GGC-3'	This study
2E1P2: 5'-CTCCCTCTGGATCCGGCTCTCATTGCC CTG-3'	
Cycles 1–35: denaturation for 60 s at 94°C; annealing for 90 s at 58°C; extension for 120 s at 72°C.	
Product: 480 bp	

Cytochrome P-450 2E1-cam C Chimera

The cytochrome 2E1 DNA (1.6-kb *Eco*RI fragment) cloned in the bluescript SK⁻ vector was obtained from the Department of Medicinal Chemistry, University of Washington, Seattle. The approach was modification of the strategy used by Gillam et al. *(9)* and Larson et al. *(26)* that involved primers 2E1P1 and 2E1LP2 for long-template amplification of P-450 2E1 product of 1461 bp with the deletion of 21 amino acids. In addition, the primers had the restriction sites for *Eco*RI and *Pst*I for cohesive ligation. The native P-450 2E1 gene in SK⁻ vector was used as template DNA for PCR amplifications. PCR products were purified by low-melt agarose gel and QIAquick gel extraction kit (Qiagen).

To construct chimera 1 the cytochrome P-450 camC gene was amplified to obtain an 822-bp product using the oligomers CE1/RP. The P-450cam C gene PCR product was subjected to partial digestion with *Bam*HI to obtain a 483-bp product. The PCR-amplified 2E1 was digested with *Bam*HI-*Pst*I to

obtain a 1.1-kb product. The two products were gel purified, ligated, and then subcloned into the *Eco*RI-*Pst*I site of pMMB 206. The fragment was also ligated into the *Eco*RI-*Pst*I site of pPROEX HTa vector. The competent cells of *E. coli* DH5α were electroporated with subcloned vector DNA, plated on LB agar containing chloramphenicol (20 mg/L) or ampicillin (100 mg/L), and the clones were screened by plasmid DNA preparation.

Chimera 2 was constructed in the *Eco*RI–*Xho*I sites of pPROEX HTa vector. A P-450 camC 483-bp fragment was obtained by restriction digestion as described for chimera 1. The PCR-amplified 2E1 gene was digested with *Bam*HI and *Hin*CII to generate a 950-bp product. To generate the 370-bp camC fragment, a 1.481-bp camC gene was PCR amplified using the primers CE1/CMCX and digested with *Hin*CII and *Xho*I. All the fragments were gel purified, ligated into the vector, and then transformed into *E. coli* DH5α cells. Clones were screened by miniplasmid preparation and the presence of pale-pink cells. Clones with correct size insert were further confirmed by PCR using CE1/CMCX primers.

Expression of Chimeric Constructs

Chimeric constructs were induced with 1 m*M* isopropylthio-β-D-galactosidase and grown at 30 and 37°C, respectively, for 24 h. The cells were harvested, sonicated in 100 m*M* potassium phosphate buffer (pH 7.4) amended with 50 m*M* KCl, and then centrifuged at 10,000*g* for 30 min to remove the cellular debris. CO-difference spectra were recorded with the clear supernatent reduced with sodium dithionite and saturated with carbon monoxide, in the range of 350–550 nm in a Perkin-Elmer UV/Vis/NIR lambda 900 spectrometer as described earlier *(15)*. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using whole-cell lysate.

Homology Modeling of Cytochrome P-450s

Homology modeling for CYP 2E1 was carried out using Ex-PDB templates of cytochrome P-450 101, BM-3, 2C17A, and 1DT6A. The initial model obtained was fine-tuned using SWISS PDB viewer v3.51 and the final model was made by using the optimize mode of SWISS-MODEL. Output files were generated in PDB format and visualized using RASMOL Windows version 2.6.

Results and Discussion

The present study explored the expression of P-450 2E1 as soluble chimeric proteins using the N-terminus alone or both the N- and C-terminus of P-450 cam. Although there is very little homology between the cytochrome P-450 camC and P-450 2E1 proteins, attempts to create chimeric proteins were solely based on their reported protein expression studies and the relevance of a heme-binding core that is common to all the known cytochrome P-450s. Earlier protein expression studies with eukary-

Fig. 1. Strategy for construction of chimeras 1 and 2. Regions in darker shading represent the fragment from cytochrome P-450 camC gene and the regions in lighter shading represent the fragment from cytochrome P-450 2E1 gene.

otic P-450s including P-450 2E1 have revealed that deletion of 20–30 amino acids from the N-terminus (which hooks the protein to the cell membrane) may allow its expression as a cytosolic protein *(6,9)*. Hence, both the chimeras were designed so that the heme-binding core and the catalytic pocket would be intact, and the camC regions would act as leader sequence for hyperexpression (Fig. 1). Since the crystal structure of P-450 2E1 is not known, homology models of chimeras 1 and 2 are based on the crystal structure coordinates of various bacterial and mammalian P450s.

Feasibility of such enzymes was determined by molecular modeling studies using SWISS MODEL and "Predict Protein" based on the known crystal structures of cytochrome P-450s cam, 2C17A, 1DT6A, and BM-3. This is based on the contention that homology models can ascertain "hot spots"

and enzyme stability. In view of the low homology between mammalian and bacterial P-450s, a model based on structures of several known enzymes is likely to be more accurate than one based on the crystal structure of a single cytochrome P-450 protein *(27)*. The model showed that the folds were appropriate for the Fe-S ligand formation, needed for catalytically active protein (Fig. 2).

Since *Pseudomonas* P-450 camC gene encodes a protein of 415 amino acids (46.668 kDa), the cytochrome P-450 2E1 gene was reconstructed by replacing 145 amino acids of the N-terminus with 130 amino acids from the N-terminus of P-450 camC (Fig. 1). The chimeric P-450 2E1 gene (chimera 1) was then subcloned into the broad host range vector, pMMB206 (Fig. 3A). The broad host range vector pMMB 206 was selected for cloning because of its transmissibility and stability in *Pseudomonas*. The BHRV pMMB 206 has the *pm* promoter (TOL plasmid) for the expression of genes under natural conditions, but hyperexpression of genes is not possible owing to chloramphenicol resistance. Hence, hyperexpression and characterization of proteins was carried out using pPROEX HTa vector (Fig. 3B). The gene was expressed in *E. coli* DH5α, as evident from the light-pink cell pellets and the presence of 55- and 60-kDa protein bands in SDS-PAGE (Fig. 3C). The N-terminus and the active core are more or less similar in both structures, but the extra 50 amino acids in the C-terminus probably increases the tertiary structure of the protein, as evident from the CO-difference spectra of the chimera 2 protein. A definite soret peak at 450 nm is clearly observed in chimera 2, whereas in chimera 1 the peak is observed in the 443-nm region, probably owing to low protein stability and degradation (Fig. 4).

Since substrate binding structurally perturbs P-450, it may control the uniqueness of P-450-catalyzed reactions by inducing intramolecular changes and stabilizing conformational changes *(27,28)*. Recent observations indicate that cytochrome P-450 2E1 is more conformationally homogeneous, more flexible, and has a very accommodating path active site relative to other P-450s *(29)*. In addition, it has been previously demonstrated that inactivated P-450 2E1 protein may not catalyze oxygen activation but can effectively transfer one electron to carbon tetrachloride *(8)*. These findings support our contention that cytochrome P-450 2E1 truly complements P-450 cam in terms of accommodating substrates at the active site and is an interesting template for generation of chimeric P-450s for designed bacterial consortia. Cytochrome P-450 2E1 chimeric enzymes will help in understanding the hurdles in codon usage and expression, protein stability and degradation, in vivo expression, and catalytic function in robust soil bacteria.

Our current understanding of diverse chemistry mediated by the superfamily of genes encoding cytochrome P-450 enzymes indicates the immense potential in genetically engineering P-450 enzymes. A combination of native, chimeric, and evolved enzymes would convert or activate a wide array of recalcitrant pollutants to derivatives that would be amenable to degradation by naturally occurring pathways in microbial communities in a natural environment.

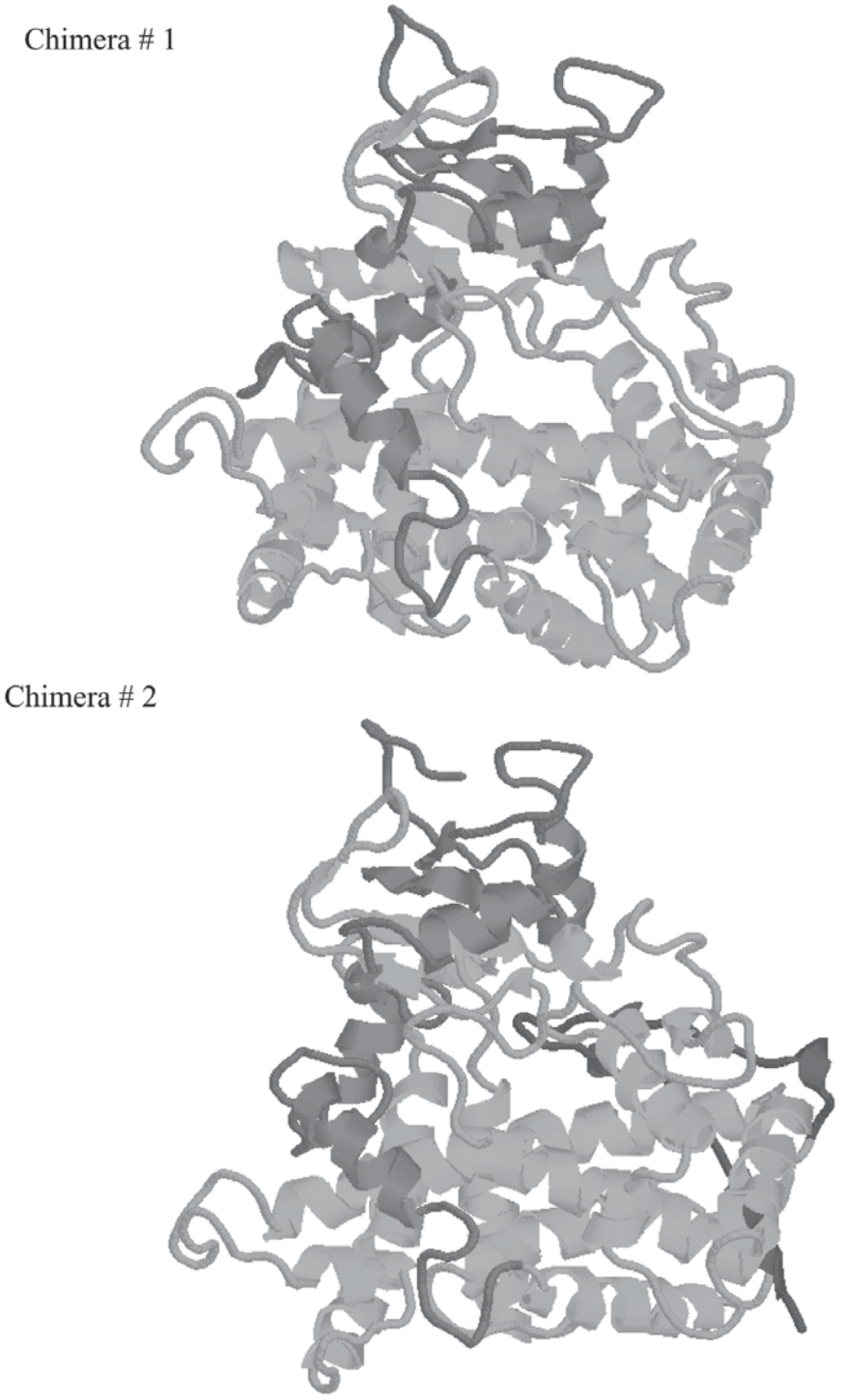

Fig. 2. Illustration of homology model of **(A)** chimera 1 and **(B)** chimera 2 protein. The region in red and blue is of cytochrome P-450 camC and regions in green are of cytochrome P-450 2E1.

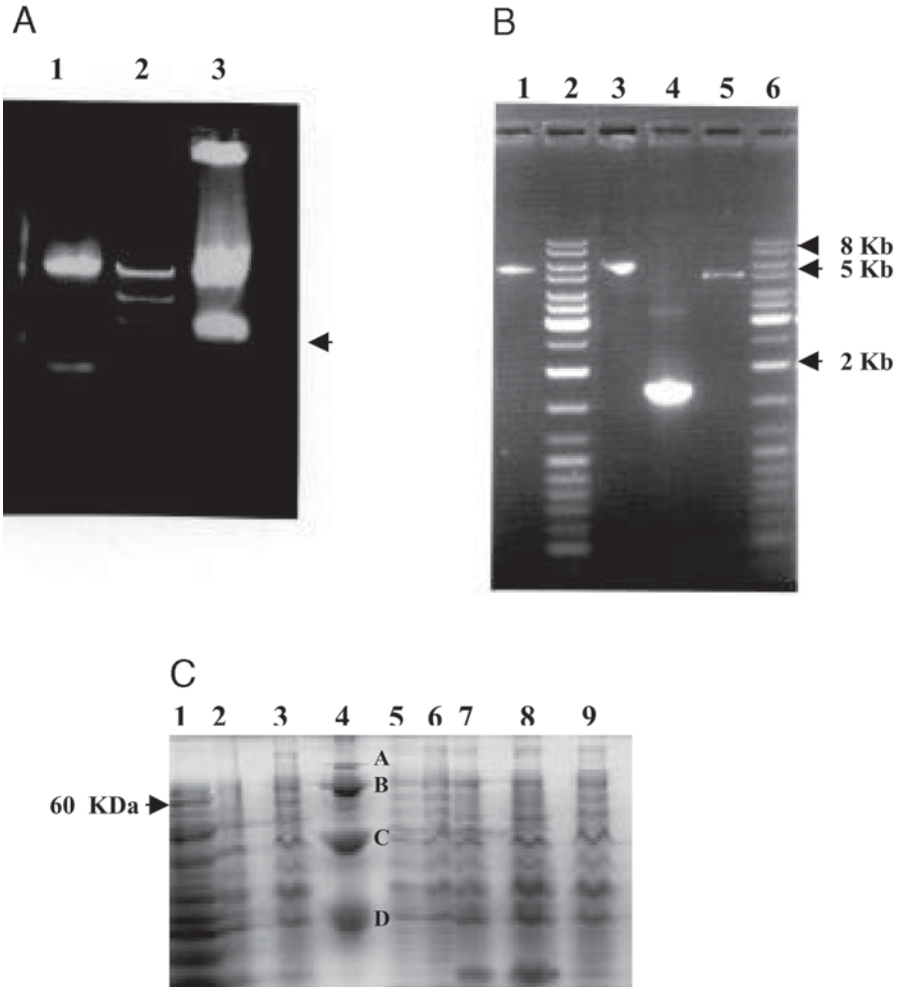

Fig. 3. Construction and expression of chimeric cytochrome P-450 2E1 possessing N-terminus or both N- and C-terminus of cytochrome P450 camC. **(A)** DNA extracted from *E. coli* DH5α possessing BHRV pMMB 206 DNA subcloned with cytochrome P-450 camC-2E1 chimeric gene 1: lane 1, pMMB 206 vector; lane 2, λ-*Hin*dIII ladder; lane 3, pMMB 206 vector with chimeric gene as insert in polylinker. **(B)** Confirmation of the clones by restriction digestion and PCR amplification: lane 1, chimera 1 gene in pPROEX HT(a) DNA linearized with *Eco*RI; lane 2, DNA marker (GeneRuler™ 1-kb DNA ladder; MBI, Fermentas); lane 3, chimera 2 gene in pPROEX HT(a) DNA linearized with *Xho*I; lane 4, PCR amplification of chimera 2 with CE1/CMCX primers; lane 5, pPROEX HT(a) linearized with *Eco*RI; lane 6, DNA marker (GeneRuler™ 1-kb DNA ladder; MBI, Fermentas). **(C)** SDS-PAGE profile of chimeras 1 and 2 cloned in pPROEX HT(a) in uninduced and induced cells of *E. coli* DH5α. The cells were mixed in gel-loading buffer, lysed by boiling for 10 min, and supernatant obtained after centrifugation at 12,000*g* was loaded on 12% gel: lane 1, induced chimera 2; lanes 2 and 3, uninduced chimera 2; lane 4, low molecular weight markers (Amersham Pharmacia) ([A] 94 kDa, [B] 67 kDa, [C] 48 kDa, [D] 30 kDa); lanes 5 and 6, uninduced induced chimera 1; lane 7, induced chimera 1; lane 8, induced pPROEX HT(a) in *E. coli* DH5α; lane 9, uninduced pPROEX HT(a) in *E. coli* DH5α.

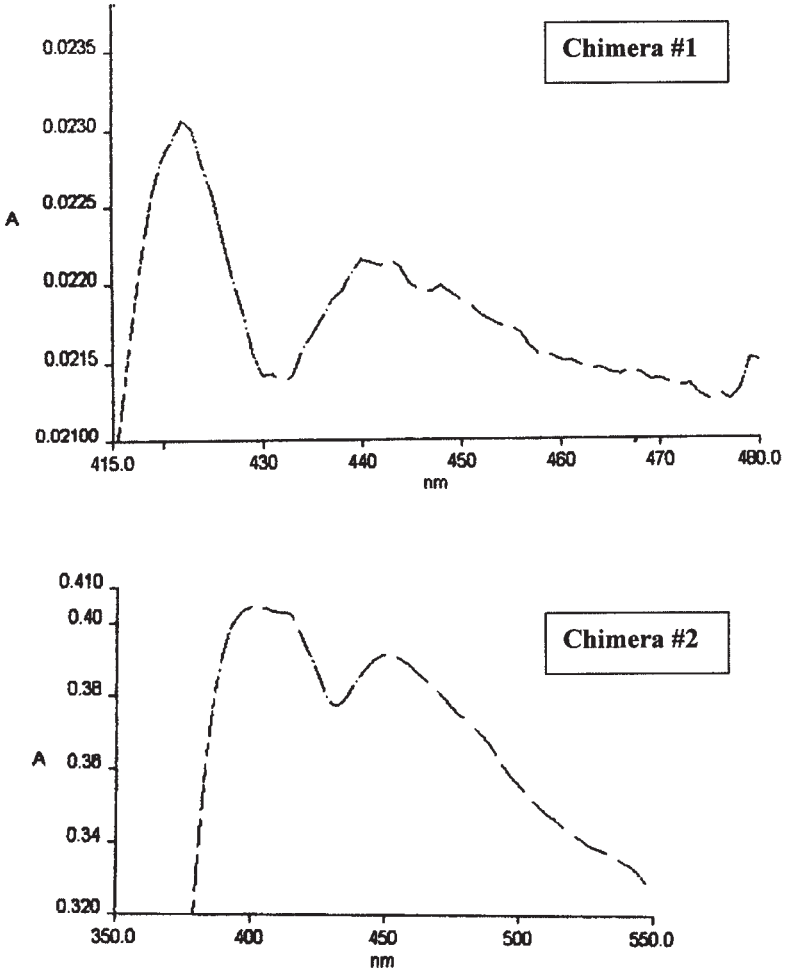

Fig. 4. Characteristic cytochrome P-450 CO-difference spectra of chimeras 1 and 2.

Conclusion

Cytochrome P-450s are widely recognized as oxygenation catalysts, but less is known on their role as reducing catalysts. Engineering of cytochrome P-450s 2E1 and 101(cam) can provide catalysts for activation of metabolic pathways for bioremediation of polyhalogenated and aromatic pollutants. Our present studies show that strategies can be developed to generate cytochrome P-450 2E1 chimeric isozymes for expression in robust soil bacteria.

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