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Surface Display of Heme- and Diflavin-Containing Cytochrome P450 BM3 in *Escherichia coli*: A Whole-Cell Biocatalyst for Oxidation

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Cytochrome P450 enzymes (P450s) are involved in the synthesis of a wide variety of valuable products and in the degradation of numerous toxic compounds. The P450 BM3 (CYP102A1) from *Bacillus megaterium* was the first P450 discovered to be fused to its redox partner, a mammalian-like diflavin reductase. Here, we report the development of a whole-cell biocatalyst using ice-nucleation protein (Inp) from *Pseudomonas syringae* to display a heme- and diflavin-containing oxidoreductase, P450 BM3 (a single, 119-kDa polypeptide with domains of both an oxygenase and a reductase) on the surface of *Escherichia coli*. The surface localization and functionality of the fusion protein containing P450 BM3 were verified by flow cytometry and measurement of enzymatic activities. The results of this study comprise the first report of microbial cell-surface display of a heme- and diflavin-containing enzyme. This system should allow us to select and develop oxidoreductases containing heme and/or flavins into practically useful whole-cell biocatalysts for extensive biotechnological applications, including selective synthesis of new chemicals and pharmaceuticals, bioconversion, bioremediation, live vaccine development, and biochip development.

Keywords: P450 BM3, oxidation, whole-cell biocatalyst, surface display

Cytochrome P450 enzymes (P450s) are remarkably diverse oxygenation catalysts found throughout nature. P450s constitute a large family of enzymes of particular interest to the pharmaceutical and chemical industries [6]. The total count of P450s is >11,200, from all classes of life including archaebacteria, fungi, plants, and animals (see http://drnelson.utmem.edu/CytochromeP450.html). The *Bacillus* P450 BM3 (CYP102A1) is a bacterial P450 system in which an oxygenase domain is fused to a mammalian-like diflavin NADPH-P450 reductase (CPR) domain in a single polypeptide. P450 BM3 is a versatile hydroxylase with a demonstrated diverse applicability [3, 5] and an established biotechnological relevance [6]. Specifically, it is interesting because of its possibility to develop as a humanized bacterial monooxygenase [13, 14, 25].

In spite of the potential use of P450s in the various biotechnological fields, considerable challenges must be overcome before the enzymes will become commonly used. Intrinsically, these enzymes are not very active and exhibit poor stability. Moreover, several steps are necessary for their purification. However, P450s, expressed in the surface of microorganisms, could be used as whole-cell biocatalysts, alone and without any purification.

The display of heterologous proteins on the surface of living cells can be used in a variety of biotechnological applications. Many peptides and proteins have been surface-displayed on microbes [4, 21, 23], viruses [1], and yeast [18]. Most cell-surface display systems are limited in the size of foreign proteins that can be expressed. Proteins smaller than 80 kDa can be displayed on bacterial cell surfaces [21, 24]. A display of bovine adrenodoxin [9, 10] containing [2Fe–2S] and rat CPR [24] containing FMN and FAD on the surface of *E. coli* have recently been reported, respectively. The display of heme-containing proteins on the bacterial surface has not yet been reported.

Here, we report the development of whole-cell biocatalysts from the creation of fusion proteins between ice-nucleation protein (Inp) and P450 BM3 for the functional display of oxidoreductases on the cell surface. We have used a surface expression vector (pSD) containing the gene fragments of

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the N and C termini of Inp, with most of the central repeating domain (CRD) of Inp deleted. The Inp-based system has been successfully used for C-terminal fusion proteins with foreign proteins, including enzymes [2, 8, 11, 12], viral antigens [16], and enzyme library [15], for display on the cell surface. The gene of P450 BM3 was inserted into this vector, and was displayed on the surface of *E. coli* JM109. We demonstrated the successful display of P450 BM3 containing complex prosthetic groups (a heme and diflavin) on the cell surface by flow cytometry, electron microscopy, and enzymatic activity assays. The activities were demonstrated using protein substrates, antibodies, or chemicals that were incapable of penetrating into the membrane.

Material and Methods

Bacterial Strains

Escherichia coli DH5α F^- ϕ80d*lacZ*ΔM15 Δ(*lac*ZYA-argF) U169 *deoR recA*1 *endA*1 *hsdR*17 *phoA supE*44 λ^- *thi-1 gyrA*96 *relA*1 and JM109 *recA1 endA*1 *gyrA*96 *thi-1 hsdR* 17 *supE*44 *relA*1 Δ(*lac-proAB*)/F'[*traD*36 *proAB*$^+$ *lac* I^q *lacZ*ΔM15] were used as the recombinant and the expression hosts, respectively.

Plasmids

The original plasmid, pSD, which contains N-terminal and C-terminal regions of the whole Inp gene (*inaK*, Genbank Accession No. AF013159) without CRD, was used to construct a surface expression vector [11]. The pSD, referred to as the null vector, was used as a control for all of our experiments. DNA sequences encoding the P450 BM3 were amplified from the pCWBM3 plasmid, which was kindly provided by Frances H. Arnold (California Institute of Technology, Pasadena, CA, U.S.A.).

Construction of the Surface Display Vectors

Plasmid pSDBM3 for the InpNC–P450 BM3 fusion protein was constructed by PCR amplification using pCWBM3 as a template and two primers, 5'-AAG**CCCGGG**ATGACAATTAAAGAAATGC CTCA-3' and 5'-TGC**GCTAGC**TTAATGATGATGATGATGCCCA GCCCACACGTCT-3'. PCR-amplified 3.2-kb DNA fragments encoding the P450 BM3 were digested with *Xma*I and *Nhe*I and were then inserted into the same enzyme-digested pSD vector. pSDBM3 was used throughout this work. pSDBM3 directs the expression of fusion proteins consisting of the N- and C-terminal regions of the Inp open reading frame and the target genes (P450 BM3) with the initiator, methionine. Expression of the plasmid was under the control of the *tac* promoter. *E. coli* JM109 containing pSD was used a negative control throughout the experiment for activity assay, spectral analysis, and flow cytometric analysis.

Culture Conditions

The expression plasmid pSDBM3 and the null vector were used to transform *E. coli* JM109 by electroporation, as described previously [20]. Single colonies of *E. coli* JM109 containing pSDBM3 were streaked on LB/ampicillin (100 μg/ml) cultures. To express the fusion proteins, starter cultures were incubated overnight at 25°C with shaking at 180 rpm and then diluted 1:100 into LB/ampicillin (100 μg/ml) medium containing additives (1 mM thiamine and 0.5 mM δ-aminolevulinic acid hydrochloride, and 25 μl/ml trace elements). Expression was induced with 1 mM IPTG when the culture reached OD_{600} of 0.4. The cells were cultivated overnight with shaking at 180 rpm at 25°C. The *E. coli* cells harboring each expression plasmid were grown overnight at 25°C, and expression of hybrid proteins was induced with IPTG. Induced cells were viable during prolonged incubation of over 24 h in the stationary phase.

Flow Cytometric Analysis

For immunofluorescence staining, *E. coli* JM109 (pSDBM3) and *E. coli* JM109 (pSD) cells were collected and washed three times with phosphate-buffered saline (PBS). The washed cells were resuspended in 1 ml of PBS, fixed with paraformaldehyde solution [0.5% (w/v)], and stored at 4°C for flow cytometric analysis. The fixed cells were resuspended in 1 ml of PBS solution containing 1% skim milk and purified rabbit anti-Inp antibody (1:25). Incubation on ice for 30 min was sufficient to allow the primary antibody to bind to its antigen. After being washed three times with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG goat antibody (1:50) (Sigma, St. Louis, MO, U.S.A.) for 30 min on ice, washed five times with PBS, and fixed with paraformaldehyde solution [0.5% (w/v)]. These cells were examined under a FACScan flow cytometer (Becton Dickinson, Oxnard, CA, U.S.A.).

Spectroscopy

Absorption spectra were recorded with a Shimadzu UV-1601 spectrophotometer (Shimadzu Corporation, Tokyo, Japan). P450 concentrations of cells and purified enzymes were quantitated by Fe^{2+}-CO *versus* Fe^{2+} difference spectroscopy [19] using an extinction coefficient of 91 $mM^{-1}\cdot cm^{-1}$ at ambient temperature. Reduction of the P450s was achieved by adding a few grains of solid sodium hydrosulfite or by adding an NADPH-generating system (5 mM glucose-6-phosphate, 1 mM $NADP^+$, and 1 international unit of yeast glucose-6-phosphate dehydrogenase/ml; final concentrations) to buffered whole-cell solutions. Formation of the carbon monoxide complexes of P450 BM3 was achieved by slowly bubbling carbon monoxide gas into the reduced enzyme solutions for approximately 1 min. Difference spectra were obtained by subtraction of the spectrum of the reduced form from that of the carbon monoxide complex form.

Estimation of Binding Constants by Spectral Titration

Because P450 BM3 is predominantly low spin, the usual binding spectral titrations were used to determine dissociation constants (K_s) for the indicated substrate, as shown previously [17]. UV/visible absorption spectra were recorded over 350–500 nm using quartz cuvettes of 1-cm path-length. Generally, the concentration of JM109 (pSDBM3) used was 0.5 μM in 1 ml of assay buffer at 25°C. Because cell viability is compromised by freezing/thawing, the binding assays were performed on the same day of culture. The decrease in absorbance of the low-spin state at 421 nm and the increase in absorbance of the high-spin species at 387 nm were monitored upon addition of substrate. The stocks of fatty acids and *p*-nitrophenoxydodecanoic acid (12-pNCA) were prepared freshly in isopropanol and dimethyl sulfoxide (DMSO), respectively. When the fatty acid was added to the sample cuvette, the same volume of solvent was added to the reference cuvette. The organic solvent concentration did not exceed 1.5% (v/v). Binding data were analyzed

by nonlinear regression using GraphPad Prism (GraphPad Prism Software Inc.).

Enzymes and Antibodies

P450 BM3 was expressed in the cytosol of the *E. coli* JM109 (pCWBM3) cells, purified as described elsewhere [17], and used for the comparison of surface-displayed P450 BM3 on the cell. The antibodies against Inp were raised from the rabbit as described previously [11].

Enzymatic Activity Assays and Whole-Cell Bioconversion

The cultures of *E. coli* JM109 (pSDBM3) were grown in LB medium for 24 h at 25°C, harvested by centrifugation, and washed three times with PBS. The washed cells were resuspended in PBS solution to a calculated final OD_{600} of 50. The cells were used for NADPH oxidation and 12-pNCA hydroxylation assays for activity assay of P450 BM3. P450 BM3 displayed on the cell surface (or purified enzyme) was pre-incubated in the presence of each substrate (200 μM) for 5 min at 25°C. Reactions were initiated by the addition of 200 μM NADPH, and the decrease in A_{340} was monitored for the first 10 s. Rates of NADPH oxidation were calculated using ε_{340}=6.22 $mM^{-1} \cdot cm^{-1}$ for NADPH. Kinetic parameters were determined using 12-pNCA as a typical substrate for P450 BM3 [22]. 12-pNCA was synthesized as previously described [22]. The reaction mixtures consisted of 0.5 μM P450 BM3 (surface-displayed or purified enzyme), 100 mM potassium phosphate (pH 7.4), and an NADPH-generating system (5 mM glucose-6-phosphate, 1 mM $NADP^+$, and 1 international unit of yeast glucose-6-phosphate dehydrogenase/ml; final concentrations), and varying concentrations of 12-pNCA (0.10–100 μM) in a total volume of 1.0 ml. After incubation for 20 min at 37°C, the reaction was stopped by the addition of 1.0 ml of cold methanol. The product was measured at 410 nm, as described previously. Kinetic parameters (K_m and k_{cat}) were determined using nonlinear regression with the GraphPad Prism software (San Diego, CA, U.S.A.).

Other Procedures and Assays

Oligonucleotides used for plasmid construction were provided by Genotech (Daejeon, Korea). Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). Nucleotide sequencing was performed in the Basic Science Research Institute (Gwangju, Korea).

RESULTS

Display of P450 BM3 on the Cell Surface of *E. coli*

For display of P450 BM3 on the cell surface, we constructed a fusion protein of InpNC and P450, as described in Materials and Methods. The expression of the fusion protein was directed from a surface display vector, pSD [11, 16]. We verified the functional display of P450 BM3 on the surface of *E. coli* JM109 by specific activity assay of displayed enzymes and flow cytometric analysis. To reduce the heme of the enzyme, NADPH, which cannot penetrate into the membrane, was externally added to the medium with expressed P450 BM3 (Fig. 1).

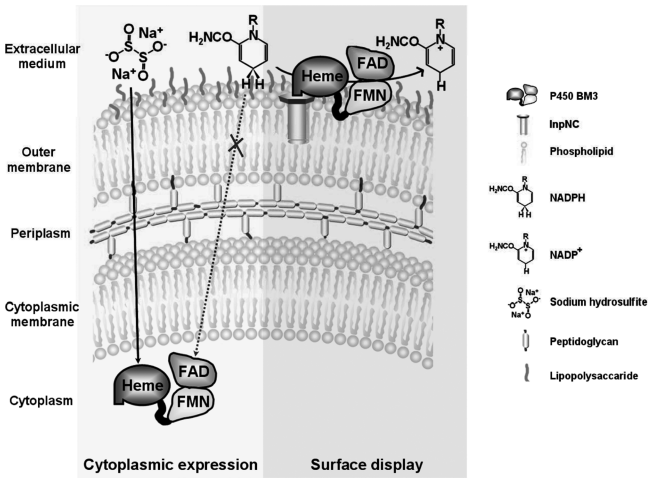

Fig. 1. Classification of P450 BM3 enzymes expressed on the microbial cell surface or in the cytosol.
NADPH cannot penetrate into membranes, but sodium hydrosulfite is capable of entering the cytosol. If P450 BM3 can be reduced by NADPH and sodium hydrosulfite, respectively, to show CO-difference spectra, then the enzymes are located on the cell surface but not in the cytosol.

If P450 BM3 is expressed in the cytosol or periplasm, NADPH cannot reduce the heme. P450 BM3, which was expressed in the cytosol using the pCW vector, did not show CO-difference spectra in the presence of externally added NADPH (Fig. 2). However, the P450 BM3 in the cytosol showed the typical CO-difference spectra when it was reduced by sodium hydrosulfite, which is capable of entering into the cytosol. When the P450 BM3 expressed on the cell surface was reduced by NADPH, it showed a typical CO-difference spectrum of P450 without any apparent P420 peak (Fig. 2). However, the extent of P450 BM3 reduction by NADPH was lower than that produced by sodium hydrosulfite. When purified P450 BM3 was

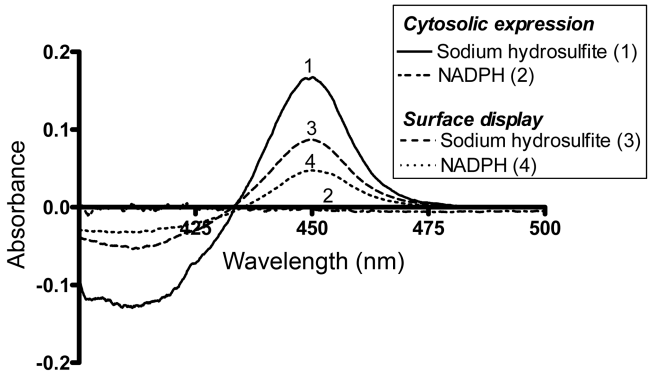

Fig. 2. Functional display of P450 BM3 on the cell surface of *E. coli* JM109.
P450 BM3 expressed in the cytosol was reduced by sodium hydrosulfite, but not by NADPH. P450 BM3 expressed on the cell surface could be reduced by NADPH or sodium hydrosulfite, respectively.

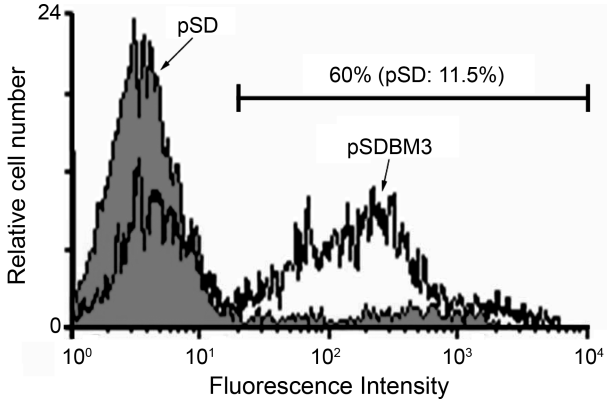

Fig. 3. Surface localization of P450 BM3 on recombinant *E. coli* JM109 cells.
Flow cytometric analyses of *E. coli* cells expressing P450 BM3 on the cell surface were performed by staining with FITC-labeled anti-rabbit IgG. The percentage of *E. coli* cells expressing P450 BM3 was evaluated.

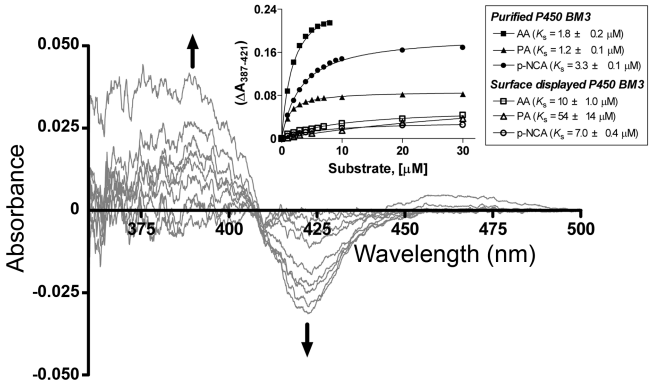

Fig. 4. Spectral binding titration of arachidonic acid (AA) to P450 BM3 on the cell surface.
Sample and reference cuvettes contained P450 BM3 (0.5 μM) in 100 mM potassium phosphate buffer (pH 7.4); arachidonic acid was added only to the sample cuvette. The inset shows the plot of concentration of arachidonic acid versus $\Delta A_{387-421}$. Binding titrations of palmitic acid (PA) and 12-pNCA were also performed with surface-displayed P450 BM3 and compared with those with purified P450 BM3.

reduced by NADPH, the extent of reduction by NADPH was approximately 2/3 of that of sodium hydrosulfite. These results clearly demonstrate that P450 BM3 is expressed on the cell surface of *E. coli* cells with full functionality.

The expression of P450 BM3 on the surface of *E. coli* was also demonstrated by immunocytochemistry. The *E. coli* JM109 cells expressing P450 BM3 were fluorescently stained with antibodies against Inp and fluorescein isothiocyanate (FITC)-labeled secondary antibody. The stained cells were subsequently analyzed by flow cytometry. Increased fluorescence was observed in cells harboring pSDBM3, but not pSD itself (Fig. 3). All of these experiments taken together provide strong evidence that InpNC protein is capable of displaying P450 BM3 on the cell surface.

Surface-Displayed P450 BM3 as Whole-Cell Biocatalysts
We also examined whether or not P450 BM3 displayed on the cell surface can be used to determine the dissociation constant (K_s) for substrates arachidonic acid (AA), palmitic acid (PA), and 12-pNCA without cell-breakage or any purification steps. Titration of surface-displayed P450 BM3 with AA, PA, and 12-pNCA yielded classic "Type I" difference spectra with K_s=10 μM, 54 μM, and 7 μM, respectively (Fig. 4). The K_s values of the substrate were higher than those obtained with purified P450 BM3. This discrepancy may be the result of the effective concentration of the substrate in solution, as the substrate may prefer to partition into the hydrophobic environment of the membrane. This result suggests that, by measuring $\Delta A_{387-421}$, the surfaced-displayed P450 BM3 can be directly used to screen ligands for the enzyme, such as substrates, inhibitors, or stimulators, which can bind to the enzyme to cause a shift in the spin equilibrium of P450 BM3 toward high-spin.

Subsequent to the addition of NADPH to the medium containing the surface-displayed P450 BM3 in the presence of the substrates lauric acid (LA), AA, PA, 12-pNCA, the decrease of A_{340} was measured to determine the rate of NADPH oxidation. The oxidation rates were usually lower than those of purified P450 BM3 (Fig. 5). This result is consistent with the increase of the K_s value of substrates (Fig. 4). Kinetic parameters of 12-pNCA oxidation by surface-displayed P450 BM3 were also determined and compared with those obtained with purified P450 BM3 (Fig. 6). Although the k_{cat} value of surface-displayed P450 BM3 was approximately 65% of the purified enzyme, the K_m value was similar to that of purified P450 BM3.

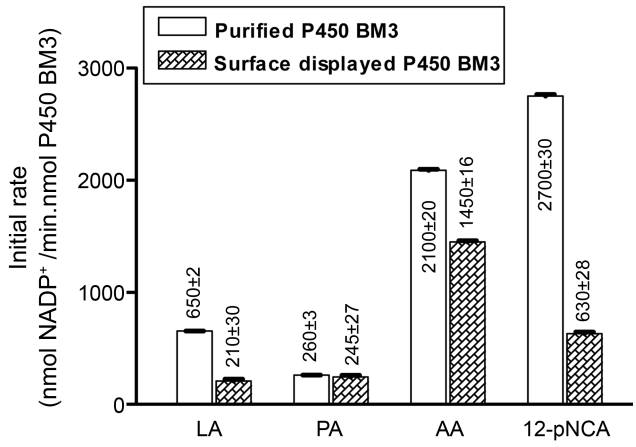

Fig. 5. Comparison of NADPH oxidation catalyzed by P450 BM3 in the presence of fatty acid substrates.
LA, lauric acid; PA, palmitic acid; AA, arachidonic acid; 12-pNCA, *p*-nitrophenoxydodecanoic acid.

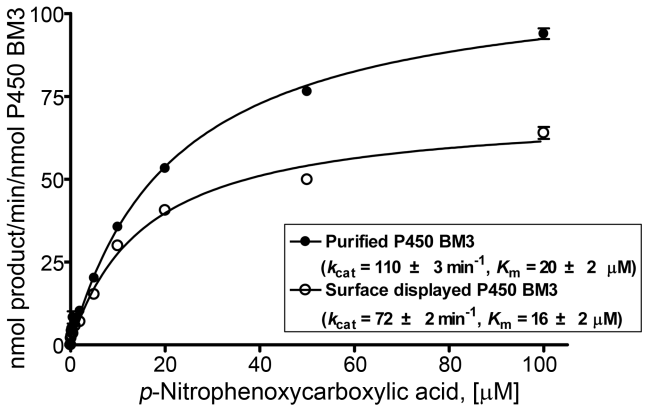

Fig. 6. Kinetic parameters (K_m and k_{cat}) of 12-pNCA hydroxylation by purified P450 BM3 or surface-displayed P450 BM3.
The rates of product formation were determined with varying concentrations of 12-pNCA (0.10–100 μM).

DISCUSSION

P450s are developing as industrially useful biocatalysts [3, 5, 6]. Although recent developments in this field provide new perspectives with respect to exploiting the P450s in biotechnological applications, many issues remain to be resolved. From a practical point of view, the great potential of P450s is limited by their functional complexity, low activity, limited stability, and low expressionability. Large amounts of active human P450 enzymes are necessary for studying drug metabolism and toxicology. However, even in recombinant microorganisms, it is not easy to express and obtain active human P450 [7]. In this context, methods of directed evolution have proven to be one of the more powerful tools for improving the utility of P450s for industrial applications [5].

Here, we have demonstrated that it is possible to achieve the surface display of *Bacillus* P450 BM3 by fusing it to a version of the Inp gene, which lacks CRD. Our results are the first to report microbial cell-surface display of a heme- and diflavin-containing enzyme. Furthermore, the heme- and diflavin-containing P450 BM3 (119 kDa) has a higher molecular mass than any proteins previously displayed on the cell surface of bacteria [21, 23]. The surface localization and functionality of fusion proteins were verified by flow cytometry and measurement of enzymatic activities. In this report, P450 BM3 is initially secreted to the outer surface by Inp and thereby immobilized, resulting in whole-cell biocatalysts genetically coating heme- and diflavin-containing P450 BM3 on the surface of *E. coli*.

The Inp system immobilizing the P450 BM3 on the surface of *E. coli* will allow us to develop practically useful whole-cell biocatalysts. There are several advantages of P450 BM3 expression on the cell surface over internal expression in the cytosol of *E. coli*. One of the technical problems in whole-cell biocatalysts is the mass-transfer limitation, because the cell membranes and cell wall act as permeability barriers. Because substrates of P450 BM3 are usually not soluble in water, process productivity would be limited for the enzymes expressed in the cytosol. The more innovative solution to the mass-transfer limitation problem would be to express the P450 BM3 on the bacterial surface. Furthermore, when their library is displayed, it provides a high-throughput screening tool for directed evolution of P450 BM3 enzymes. For the directed evolution of the P450 BM3 by random mutagenesis, high active mutants can be selected by flow cytometry. Several fluorescent products produced by P450 enzymes are known [25].

The Inp–P450 BM3 fusion proteins were targeted to the cell surface because the P450 BM3 was designed to fuse with Inp, which is located at the outer membrane. Earlier studies regarding Inp and Inp-fusion proteins indicate that some of the fusion proteins with Inp are located in the cytosol [11, 24]. In the case of Inp–rat NADPH P450 reductase fusion proteins, only 42% of the expressed proteins were targeted to the outer membrane and the rest were located in the cytoplasm, periplasm, and inner membrane [24]. Therefore, some portion of the Inp–P450 BM3 fusion proteins would be located in the cytoplasm, inner membrane, and periplasm. However, the precise secretion mechanism of heme- and flavin-containing P450 BM3 using Inp from *Pseudomonas syringae* as an anchor molecule has not yet been well studied in detail.

In conclusion, this genetic system immobilizing the P450s or related reductases on the surface of a living microorganism as a whole-cell biocatalyst should allow us to overcome the aforementioned obstacles for a wide variety of biotechnological applications, including combinatorial biocatalysis for selective synthesis of new chemicals and pharmaceuticals (drugs), bioconversion, bioremediation, live vaccine development, and biochip development. With the intent of using enzyme-coated cells for various biotechnological applications, Inp-mediated surface display for P450 BM3 could expose a new dimension in the development of whole-cell catalysts. We ensure that this report on an *in vivo* immobilization method of P450 BM3 might open a new way of applying heme- and flavin-containing proteins on the industrial scale.

Acknowledgments

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