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Efficient Biotransformations Using *Escherichia coli* with *tolC acrAB* Mutations Expressing Cytochrome P450 Genes

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We report here some efficient biotransformations using *Escherichia coli* strains with disruptions for the AcrAB-TolC efflux pump system. Biotransformations of compactin into pravastatin (6α-hydroxy-iso-compactin) were performed using *E. coli* strains with *tolC* and/or *acrAB* mutations expressing a cytochrome P450 (P450) gene. The production levels of pravastatin using strains with *acrAB*, *tolC*, and *tolC acrAB* mutations increased by 3.7-, 7.0-, and 7.1-fold, respectively. Likewise, the production levels of 25-hydroxy vitamin D_3 and 25-hydroxy 4-cholesten 3-one using *tolC acrAB* mutant strains expressing an individual P450 gene increased by 2.2- and 16-fold, respectively. The enhancement of this biotransformation efficiency could be explained by increases in the intracellular amounts of substrates and the concentrations of active P450s. These results demonstrate that we have achieved versatile methods for efficient biotransformations using *E. coli* strains with *tolC acrAB* mutations expressing P450 genes.

Key words: biotransformation; cytochrome P450; pravastatin; *tolC*

There is considerable interest in the development of technology for the production of commercially valuable chemicals from hydrophobic or amphiphilic compounds using biocatalysts. In particular, *regio*-specific oxidation of compounds has attracted much interest because many important chemicals can be produced by these reactions.[1] The enzymatic oxidation reactions require regeneration of the redox cofactor, for example NADH, and thus biotransformation using the whole cell is favored as a method for this biocatalysis.[2] The above necessitates the use of biotransformation systems using *Escherichia coli* expressing several oxidases, including cytochrome P450 (P450), for the development of bioproduction processes.

Biotransformations using *E. coli* expressing enzymatic genes have been performed for some other industrial bioproduction processes,[3,4] and it is commercially important to enhance the biotransformation efficiency. We previously showed that L-pipecolic acid can be produced by the biotransformation of L-lysine using *lat*-expressing *E. coli*, and the efficiency of L-pipecolic acid production was enhanced by *lysP* and *yeiE* amplification.[5] As propounded by the enzymatic kinetics theory, the enzymatic reaction rate increases as the concentration of substrate increases when enzyme quantity is kept constant and the substrate concentration is low enough. An enhancement of the ability to uptake L-lysine by overexpressing *lysP* (encoding lysine-specific permease) led to L-lysine accumulation in *E. coli* cells, and that caused efficient bioproduction of L-pipecolic acid.

Several physiological and biochemical approaches have been applied with the aim of elucidating the mechanisms of microbial resistance to solvents and antibiotics.[6] Genetic evidence suggests that the AcrAB-TolC efflux pump is involved in the solvent resistance of *E. coli* cells, and the AcrAB-TolC pump reduces the intracellular solvent concentration in *E. coli* cells exposed to solvents.[7] The antibiotic susceptibility profiles of *E. coli* strains lacking multidrug efflux pump genes revealed that deletions in *acrAB* or *tolC* definitely resulted in increased susceptibility to the majority of compounds tested.[8]

The P450 enzymes constitute a large superfamily of heme proteins involved in the metabolism of a wide variety of both exogenous and endogenous compounds.[9] Usually, they act as the terminal oxidase in multicomponent electron transfer chains. All the multicomponent electron transfer chains which contain P450 are termed here P450-containing systems. P450-containing systems can be classified according to the number of their protein components.[10] Mitochondrial and most bacterial P450-containing systems have three components: an FAD-containing flavoprotein (ferredoxin reductase), an iron-sulphur protein (ferredoxin), and a P450.

One of the most famous biotransformations using P450 expressed by microorganisms is that of compactin into pravastatin, which is 6α-hydroxy-iso-compactin and a 3-hydroxy-3-methyl glutaryl CoA reductase inhibitor. Pravastatin is produced by two-step fermentation. First compactin is produced by *Penicillium citrinum*, and then it is oxidized by biotransformation using *Streptomyces carbophilus* to form pravastatin.[11,12] The P450 enzyme responsible for this oxidation, cytochrome P450 105A3 (P450sca-2), was identified and its coding gene was cloned.[13] However, no study about the biotransformation of compactin into pravastatin using a recombinant strain was reported except that using *Streptomyces lividans* expressing the P450sca-2 gene.[13]

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Abbreviations: BRM, biotransformation reaction mixture; 4C3O, 4-cholesten 3-one; OMF, outer membrane factor; P450, cytochrome P450; PMCD, 2,3,6-partially methylated-β-cyclodextrin; VD3, vitamin D_3

We report here versatile methods for efficient biotransformations of hydrophobic and amphiphilic compounds to produce their *regio*-specific oxidized products using *E. coli* strains with *tolC acrAB* mutations expressing P450 genes.

Materials and Methods

Strains. *E. coli* BLstarTol (*tolC210*::Tn*10*) was constructed by P1 transduction of the *tolC210*::Tn*10* allele from CAG12184 (provided by the *E. coli* Genetic Stock Center, Yale University) into BL21star(DE3) (Invitrogen, Carlsbad, CA). *E. coli* BLstarAcr (*acrAB*::*cat*) and BLstarTolAcr (*tolC210*::Tn*10*, *acrAB*::*cat*) were constructed by P1 transduction of the *acrAB*::*cat* allele from JA300A into BL21star(DE3) and BLstarTol, respectively.[7] The mutations were confirmed by DNA sequencing.

Streptomyces sp. TM-7 was used for the isolation of *boxAB* (Accession no. AB180845). *Pseudonocardia autotrophica* NBRC 12743 was used for the isolation of *vdh* (Accession no. AB456955). *Deactylosporangium variesporum* IFO 14104 was used for the isolation of *dvbA* (Accession no. AB442014).

DNA manipulation. Chromosomal DNAs from *Streptomyces* sp. TM-7, *Pseudonocardia autotrophica* NBRC 12743, and *Deactylosporangium variesporum* IFO 14104 were prepared using Isoplant (Nippon Gene, Tokyo). Plasmids were prepared using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). DNA ligation was performed using the ligation kit (Takara, Kyoto). All restriction enzymes were obtained from Takara. All amplifications by PCR were performed using KOD-Plus-(Toyobo, Osaka). DNA sequencing analysis was done using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The primers used in this study were synthesized by Sigma Aldorich Japan (Tokyo) and are shown in Table 1. The plasmids used in this study were constructed as follows.

A fragment of *aciC* derived from pDolABC was ligated into the *Bam*HI-*Hin*dIII site of pETduet-1 (Novagen, San Diego, CA).[14] Then, a DNA fragment of *boxB* was amplified from *Streptomyces* sp. TM-7 chromosomal DNA using primer 1 and primer 2. Next, a DNA fragment of the *boxA* was amplified from *Streptomyces* sp. TM-7 chromosomal DNA using primer 3 and primer 4, digested with *Nde*I and *Xho*I, and ligated into the *Nde*I-*Xho*I site of this plasmid. The resulting plasmid was named pCP101.

A DNA fragment of *aciB* from pDolABC was digested with *Nco*I and *Bam*HI and ligated into the *Nco*I-*Bam*HI site of pETduet-1. The *aciC* fragment was amplified from pDolABC using primer 5 and primer 6, digested with *Bam*HI and *Hin*dIII, and ligated into the *Bam*HI-*Hin*dIII site of this plasmid. Then the 2nd *T7*-promoter and the *Nde*I site in this plasmid were removed by digestion with *Eco*RV and *Not*I, and this DNA fragment was blunted and self-ligated. Then the DNA fragment containing the SD-sequence and the *Nde*I site was amplified using primer 7 and primer 8, digested with *Bgl*II and *Bam*HI, and ligated into the *Bgl*II-*Bam*HI site of the plasmid. The resulting plasmid was named pEN1122.

A DNA fragment of the N-terminal 150 bp of *aciA* was amplified from pDolABC using primer 9 and primer 10, and digested with *Nde*I and *Spe*I. A DNA fragment of *dvbA* was amplified from *Dactylosporangium variesporum* IFO 14104 chromosomal DNA using primer 11 and primer 12, and digested with *Spe*I and *Bam*HI. The resulting fragments were then ligated into the *Nde*I-*Bam*HI site of pEN1122, and the resulting plasmid was named pEN1123.

A DNA fragment of *vdh* was amplified from *Pseudonocardia autotrophica* NBRC 12743 chromosomal DNA using primer 13 and primer 14, and digested with *Spe*I and *Bgl*II. This fragment was then ligated into the *Spe*I-*Bam*HI site of pEN1123, and the resulting plasmid was named pEN7373.

Table 1. Primers Used in This Study

Primer number	Sequence (5′ → 3′)
primer 1	CAGCCATGGGTGTGACGGCCGACCGGGAGGT
primer 2	CACGGATCCTACTCGACGACGCGTACCGCGCC-GGA
primer 3	GGACATATGACCGAGACCGTTACGACGCCC
primer 4	TCGCTCGAGTCACCAGGTGACCGGGAGTTC
primer 5	CATGGATCCTGAACTGAGTGAATTGATATGCAA
primer 6	CCCAAGCTTCTACCCCATCAACGCCTGTACC
primer 7	GTAAGATCTAAATAAGGAGGAATAACATATGG-CGCTGACCACCACCGGCA
primer 8	CCCGGATCCCGGTGCCGGTGGTGGTCAGCGCCA
primer 9	TAACATATGAACTCAGTCGCAGAAATTTTTGA
primer 10	CGAACTAGTGGTCACTTGACCATGCAAAAACT
primer 11	CCGACTAGTACCGAAACGCTGTACCCCGAG
primer 12	CCTGGATCCTCATGAGAACGTCACCGGCAG
primer 13	ACCACTAGTGCGCTGACCACCACCGGCACCG
primer 14	GGGAGATCTTCAGGCGCTGCGCGGCCCCATC

Biotransformation of compactin. *E. coli* BL21star(DE3), BLstarAcr, BLstarTol, and BLstarTolAcr were transformed with pCP101, and each strain was cultivated at 25 °C with shaking in P450M9-SEED broth (6.78 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 10 g/l casamino acids, 4 g/l D-glucose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM $FeSO_4$, and 20 mg/l thymine) containing 100 mg/l carbenicillin. After 24 h of cultivation, 200 µl of each culture was added into 25 ml of P450M9-Main broth (6.78 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 10 g/l casamino acids, 0.1 mM $CaCl_2$, 0.1 mM $FeSO_4$, 20 mg/l thymine, and 80 mg/l 5-aminolevulinic acid) containing 100 mg/l of carbenicillin and the Overnight Expression System (20 µl of solution I, 50 µl of solution II, and 1 µl of solution III per ml, Merck, Darmstadt, Germany). 5-Aminolevulinic acid, a precursor of heme, was added because P450 enzymes are heme-containing proteins and require heme to be expressed. After 24 h of cultivation at 25 °C with shaking at 220 rpm, the cells were collected (about 0.66 g of wet cells) and suspended in 5 ml of CV2 buffer (50 mM sodium phosphate buffer, pH 7.4, 2% (v/v) glycerol, 50 mg/l carbenicillin, and 0.1 mM isopropyl-β-D-thiogalactopyranoside). This mixture was defined as biotransformation reaction mixture (BRM). Biotransformations were started when 30 µl of 25 mg/ml of sodium compactin in water were added into 1 ml of BRM in test tubes. After 0, 2, 5, 9, or 24 h of incubation at 28 °C with shaking at 220 rpm, 1 ml of acetonitrile and 1 ml of methanol were added to each test tube and mixed. For the sample of 24 h of incubation, an additional 30 µl of the compactin solution was added after 9 h of incubation. The samples were centrifuged at 20,000 × *g* for 5 min, and 1 ml of the supernatant was recovered. Then HPLC analyses were performed as described below.

Biotransformation of vitamin D_3. *E. coli* BL21star(DE3), BLstarAcr, BLstarTol, and BLstarTolAcr were transformed with pEN7373, and BRM was prepared using these cells. Then 25 µl of 10 mg/ml of vitamin D_3 (VD3) in DMSO and 30 µl of 25% 2,3,6-partially methylated-β-cyclodextrin (PMCD) were added to 1 ml of BRM in test tubes. PMCD was added for solubilization of VD3. After 0, 2, 4, 6, 8, or 24 h of incubation at 28 °C with shaking, 2 ml of methanol was added into each test tube and mixed. These samples were centrifuged at 20,000 × *g* for 5 min, and 1 ml of the supernatant was recovered. Then HPLC analyses were performed as described below.

Biotransformation of 4-cholesten 3-one. *E. coli* BL21star(DE3), BLstarAcr, BLstarTol, and BLstarTolAcr were transformed with pEN1123, and BRM was prepared using these cells. Then 40 µl of 10 mg/ml of 4-cholesten 3-one (4C3O) in methanol and 30 µl of 25% PMCD were added to 1 ml of BRM in test tubes. PMCD was added for solubilization of 4C3O. After 0, 2, 4, 6, 8, or 24 h of incubation at 28 °C with shaking, 2 ml of methanol was added to each test tube and this was mixed. These samples were centrifuged at 20,000 × *g* for 5 min, and 1 ml of the supernatant was recovered. Then HPLC analyses were performed as described below.

Determination of the intracellular amounts of substrates. The intracellular amounts of substrates were determined by method similar to that described previously.[7] Briefly, *E. coli* BL21star(DE3), BLstarAcr, BLstarTol, and BLstarTolAcr were cultivated at 25 °C with

shaking in P450M9-SEED broth. After 24 h of cultivation, 200 µl of the culture was added to 25 ml of same media. After 24 h of cultivation at 25 °C with shaking at 220 rpm, the cells were collected (about 0.64 g of wet cells) and suspended in 5 ml of CV buffer (50 mM sodium phosphate buffer, pH 7.4, and 2% (v/v) glycerol). Then 30 µl of 25 mg /ml of sodium compactin in water, 100 µl of 10 mg/ml of VD3 in DMSO and 80 µl of PMCD, or 20 µl of 10 mg/ml of 4C3O in methanol and 30 µl of 25% PMCD were added to 1.0 ml of this cell suspension in a test tube. After 4.5 h of incubation at 28 °C with shaking at 220 rpm, samples were centrifuged at 2,000 × *g* for 5 min, and the precipitates were recovered. After these precipitates were washed twice, 1 ml of CV buffer was added and the substrates were extracted as described above. Then, HPLC analyses were performed as described below.

CO difference spectral analysis. To quantitate the concentration of P450s in the *E. coli* cells, CO difference spectral analyses were performed. The CO difference spectrum of each BRM diluted by 5-fold was measured with a UV-visible spectrophotometer U-3310 (Hitachi, Tokyo) as described previously.[15] In order to calculate the concentrations of active P450s, an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ was used (concentration of active P450 (nM) = CO difference at 450 nm/91 ($mM^{-1}\,cm^{-1}$) × 10^6.

HPLC analyses. Pravastatin and compactin were analyzed by HPLC on a Chromolith Performance RP-18e (4.6 mm I.D. × 100 mm) column maintained at 40 °C using a methanol concentration gradient in water aqueous solution (containing 0.1% triethylamine and 0.1% of acetic acid) from 50% to 90% for 3 min, and 90% to 90% for 0.5 min, followed by 50% to 50% for 2.5 min at a flow rate of 2.0 ml per min. The derivatives were detected by UV-monitoring at 238 nm. Pravastatin and compactin had retention times of 1.8 min and 3.2 min, respectively.

25-hydroxy VD3 and VD3 were analyzed by HPLC on a J′ sphere ODS-H80 (4.6 mm I.D. × 75 mm) column maintained at 40 °C using an acetonitrile concentration gradient in water aqueous solution from 30% to 30% for 13 min, 30% to 100% for 1 min, 100% to 100% for 7 min, and 100% to 70% for 1 min, followed by 70% to 70% for 3 min at a flow rate of 1.0 ml per min. The derivatives were detected by UV-monitoring at 265 nm. 25-hydroxy VD3 and VD3 had retention times of 3.0 min and 9.3 min, respectively.

25-hydroxy 4C3O and 4C3O were analyzed by HPLC on a Inertsil ODS-3 (4.6 mm I.D. × 50 mm) column maintained at 40 °C using an acetonitrile concentration gradient in 0.85% phosphate aqueous solution from 40% to 100% for 5 min, 100% to 100% for 3 min, and 100% to 40% for 0.5 min, followed by 40% to 40% for 2.5 min at a flow rate of 1.2 ml per min. The derivatives were detected by UV-monitoring at 235 nm. 25-hydroxy 4C3O and 4C3O had retention times of 5.3 min and 8.1 min, respectively.

Results

Biotransformation of compactin into pravastatin

Previously, we isolated the *boxAB* gene from *Streptomyces* sp. TM-7. The *boxA* gene encodes the protein belonging to CYP105 family that showed high similarity to P450sca-2 with 76% identity. This gene was followed by *boxB* encoding a protein showing high similarity to many ferredoxins from *Streptomyces*. In order to utilize the P450-containing system including BoxA for bioproduction, we constructed pCP101 expressing P450-containing system genes composed of *boxA*, *boxB*, and *aciC* (Fig. 1A). We adopted *aciC* as the gene encoding a ferredoxin reductase, because no gene encoding ferredoxin reductases was in the neighborhood of *boxAB*, and *E. coli* expressing P450-containing system genes composed of *aciABC* showed efficient biotransformation activity of *n*-octane.[14]

Biotransformation of compactin, an amphiphilic compound, into pravastatin was performed using the *E. coli* strains with *tolC* and/or *acrAB* mutations transformed with pCP101. About 237, 867, 1,670 and 1,700 mg/l of pravastatin were produced from compactin after 24 h of incubation by biotransformation using *E. coli* BL21star(DE3), BLstarAcr, BLstarTol, and BLstarTolAcr, respectively (Fig. 2A). Accordingly, the production levels of pravastatin using strains with *acrAB*, *tolC*, and *tolC acrAB* mutations increased by 3.7, 7.0, and 7.1-fold, respectively. Almost all the compactin consumed in these biotransformations were converted into pravastatin without loss.

Biotransformation of VD3 into 25-hydroxy VD3

In order to confirm the superiority of the *E. coli* strains with *tolC* and/or *acrAB* mutations for bioproduction using P450 genes, we attempted biotransformation of VD3, a hydrophobic compound, into 25-hydroxy VD3 using a P450 gene *vdh* belonging to the CYP107 family (Fujii Y, *et al.*, unpublished results). We constructed pEN7373 expressing P450-containing system genes composed of *vdh*, *aciB* (encoding a ferredoxin), and *aciC* (Fig. 1B),[14] because no gene encoding ferredoxins or ferredoxin reductases were in the neighborhood of *vdh*. Then we performed biotransformation of VD3 into 25-hydroxy VD3 using *E. coli* strains with *tolC* and/or *acrAB* mutations transformed with pEN7373. About 96.2, 135, 187, and 216 mg/l of 25-hydroxy VD3 was produced from VD3 after 24 h of incubation with *E. coli* BL21star(DE3), BLstarAcr, BLstarTol, and BLstarTolAcr, respectively (Fig. 2B). Accordingly, the production levels of 25-hydroxy VD3 using strains with *acrAB*, *tolC*, and *tolC acrAB* mutations increased by 1.4, 1.9, and 2.2-fold, respectively. Almost all the VD3 consumed in these biotransformations were converted into 25-hydroxy VD3 without loss.

Biotransformation of 4C3O into 25-hydroxy 4C3O

We also attempted biotransformation of 4C3O, a hydrophobic compound, into 25-hydroxy 4C3O using a P450 gene *dvbA* belonging to the CYP105 family. We constructed pEN1123 expressing P450-containing system genes composed of *dvbA*, *aciB*, and *aciC* (Fig. 1C). Then, we performed biotransformation of 4C3O into 25-hydroxy 4C3O using mutant strains transformed with pEN1123. About 26.4, 79.7, 398, and 415 mg/l of 25-hydroxy 4C3O was produced from 4C3O after 24 h of incubation with *E. coli* BL21star(DE3), BLstarAcr, BLstarTol, and BLstarTolAcr, respectively (Fig. 2C). Accordingly, the production levels of 25-hydroxy 4C3O using strains with *acrAB*, *tolC*, and *tolC acrAB* mutations increased by 3.0-, 15-, and 16-fold, respectively. Almost all the 4C3O consumed in these biotransformations were converted into 25-hydroxy 4C3O without loss.

Intracellular amounts of substrates

We exposed *E. coli* cells with *tolC* and/or *acrAB* mutations to compactin and analyzed the intracellular amounts of them. The intracellular amounts of compactin in *E. coli* cells with *acrAB*, *tolC*, and *tolC acrAB* mutations increased by 7.0, 75, and 77-fold, respectively (Fig. 3A). The intracellular amounts of VD3 and 4C3O similarly increased in the *tolC* and *tolC acrAB* mutant strains, whereas decreased in the *acrAB* mutants (Fig. 3B and C). These results suggest that *E. coli*

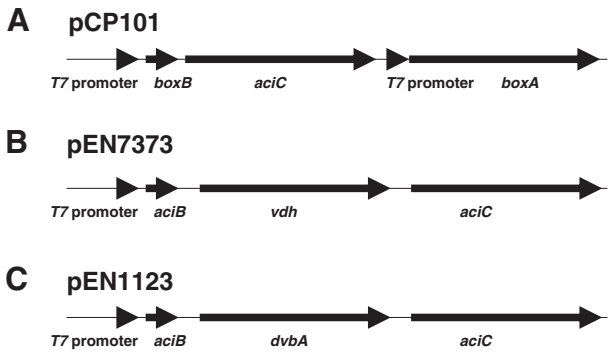

Fig. 1. P450-Containing System Genes on Plasmids.

Plasmids were constructed composed of P450-containing system genes for the biotransformation of compactin (A), VD3 (B), and 4C3O (C), respectively. Triangles show *T7* promoters. Arrows show the genes belonging to P450-containing systems. *boxA*, a P450 gene (CYP105 family) isolated from *Streptomyces* sp. TM-7; *vdh*, a P450 gene (CYP107 family) isolated from *P. autotrophica* NBRC 12743; *dvbA*, a P450 gene (CYP105 family) isolated from *D. variesporum* IFO 14104; *boxB*, a ferredoxin gene isolated from *Streptomyces* sp. TM-7; *aciB*, a ferredoxin gene isolated from *Acinetobacter* sp. OC4; *aciC*, a ferredoxin reductase gene isolated from *Acinetobacter* sp. OC4.

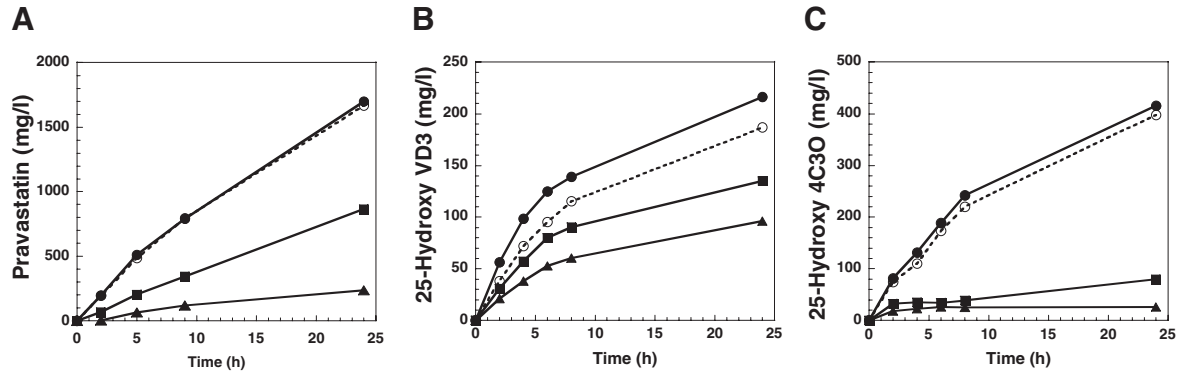

Fig. 2. Biotransformation Using *E. coli* Strains with *tolC* and/or *acrAB* Mutations Expressing P450-Containing System Genes.

Biotransformation of compactin into pravastatin (A), VD3 into 25-hydroxy VD3 (B), and 4C3O into 25-hydroxy 4C3O (C) was performed using *E. coli* BL21star(DE3) (triangles), BLstarAcr (squares), BlstarTol (open circles), and BLstarTolAcr (closed circles) individually transformed with pCP101(A), pEN7373 (B), and pEN1123 (C). After incubation at 28 °C, the amounts of products in 1 ml of reaction solution were determined. Control experiments using *E. coli* cells transformed with pETduet-1 did not convert the substrates at all. Three independent experiments were done, and the average is shown.

strains with *tolC* mutation reduced the abilities to release substrates (hydrophobic or amphiphilic compounds) and accumulated them inside the cells, and that *acrAB* mutation in the *tolC* mutant strain had additive effects. Only in the case of compactin, the *acrAB* mutation added into the wild-type strain seemed to contribute to an additional accumulation of the substrate.

CO difference spectral analyses of the P450s

In order to quantitate the concentrations of active BoxA, we analyzed the spectral characteristics of BoxA in *E. coli* cells with *tolC* and/or *acrAB* mutations transformed with pCP101. The intracellular concentrations of BoxA in the *E. coli* cells with *acrAB*, *tolC*, and *tolC acrAB* mutations increased by 11, 18, and 27-fold, respectively (Fig. 4A). Similarly, the intracellular concentrations of VDH and DvbA in *E. coli* cells increased a little with *acrAB* mutation and largely with *tolC* mutation (Fig. 4B and C). These results suggest that *E. coli* strains with *tolC* mutation efficiently accumulated P450s expressed their own. In the cases of BoxA and DvbA, the *acrAB* mutation added into the *tolC* mutant strain contributed to additional accumulation of P450s.

Discussion

Biotransformations using *E. coli* have been performed for some industrial bioproductions, and it is commercially important to enhance their biotransformation efficiency. We demonstrated here that the production level of pravastatin using the strain with *tolC acrAB* mutations increased by 7.1-fold, and about 1,700 mg/l of pravastatin was produced.

One explanation for this enhancement could be derived by the result that the intracellular amount of compactin in the *tolC acrAB* mutant strain increased by 77-fold. As propounded by the enzymatic kinetics theory, the enzymatic reaction rate increases as the concentration of substrate increases. The disruption of AcrAB-TolC efflux pump system reduces the ability to release substrates and accumulates them inside the cells, and thus enhanced oxidation by P450 might be achieved. In a water-organic solvent two-phase system, it was reported that organic solvent resistant *E. coli* showed higher biotransformation ability than the wild-type strain.[16] Other solvent-tolerant bacteria able to grow in the presence of organic solvents are also thought to be good candidates for the biotransformation of hydrophobic substrates.[17] Contrary to these general assump-

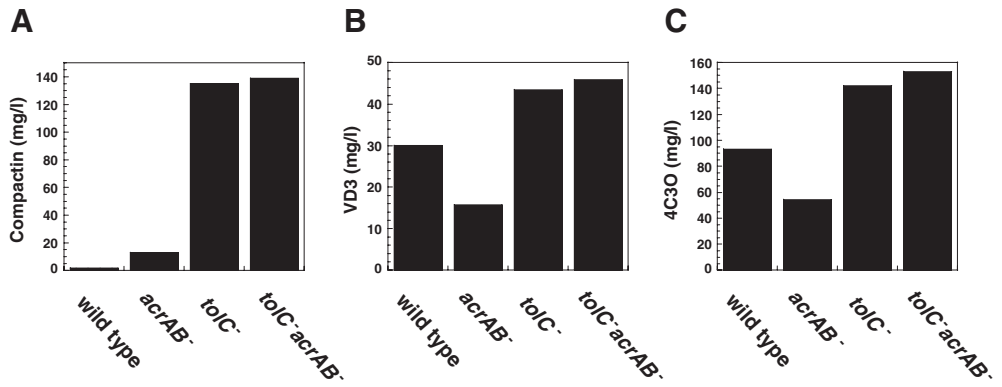

Fig. 3. Entry of Substrates into *E. coli* Cells with *tolC* and/or *acrAB* Mutations.

E. coli BL21star(DE3) (wild type), BLstarAcr (*acrAB*$^-$), BlstarTol (*tolC*$^-$), and BLstarTolAcr (*tolC*$^-$ *acrAB*$^-$) were incubated in 1 ml of media with compactin (A), VD3 (B), and 4C3O (C) individually, and the amounts of these substrates in these cells were determined.

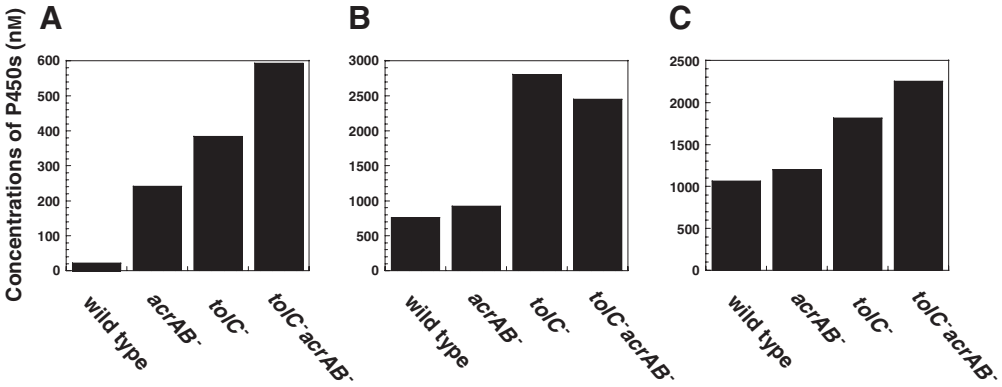

Fig. 4. CO Difference Spectral Analyses.

The CO difference spectrum of *E. coli* BL21star(DE3) (wild type), BLstarAcr (*acrAB*$^-$), BlstarTol (*tolC*$^-$), and BLstarTolAcr (*tolC*$^-$ *acrAB*$^-$) transformed with pCP101 (A), pEN7373 (B), and pEN1123 were analyzed individually. Then the concentrations of active P450s (BoxA (A), Vdh (B), and DvbA (C)) in cells were calculated. Three independent experiments were done, and the average is shown.

tions, our strains with *tolC acrAB* mutations showed higher biotransformation ability than the wild-type strain in water-phase systems.

Another explanation for the enhanced biotransformation of compactin is that the intracellular concentration of BoxA in *E. coli* cells with *tolC acrAB* mutation increased by 27-fold. Recently, it was reported that the TolC-dependent efflux system is involved in the exclusion of porphyrin(ogen)s, including heme.[18] The *tolC* mutant strains reduce the ability to release not only substrates but also heme and accumulate them inside the cells. Because P450 enzymes are heme-containing proteins, accumulation of heme in the cells might lead to increased expression of P450s and thereby enhanced oxidation might be achieved.

Some studies have reported on the biotransformation of compactin into pravastatin using *Streptomyces*. The highest production level of pravastatin was 340 mg/l from 750 mg/l of compactin for 24 h.[19] In the *Streptomyces*-system, only about 60% of compactin was converted into pravastatin and about 40% of compactin was metabolized into other chemicals, possibly due to oxidation activities by endogenous P450s. This is one of the most serious problems in the industrial production of pravastatin, because compactin is the most expensive substance in the material cost. On the other hand, almost the compactin consumed in our *E. coli*-system was converted into pravastatin without loss, possibly because *E. coli* had no endogenous P450s. Thus our *E. coli*-system has an advantage over other *Streptomyces*-systems.

We also report here that the production levels of 25-hydroxy VD3 and 25-hydroxy 4C3O using strains with *acrAB*, *tolC*, and *tolC acrAB* mutations increased, and especially those using the *tolC acrAB* mutant strains increased by 2.2 and 16-fold, respectively. These results demonstrate the versatility of our methods for efficient biotransformations using *E. coli* strains with *tolC acrAB* mutations expressing P450 genes. The biotransformation efficiencies of VD3 and 4C3O using the *acrAB* mutant strain were higher than those using the wild-type strain in spite of decreases in the intracellular amounts of substrates, suggesting that the increased accumulation of P450s in the *acrAB* mutant contribute to enhanced biotransformation. The biotransformation efficiency of VD3 using the *tolC acrAB* mutant strain was higher than that using *tolC* mutant in spite of decreases in the intracellular amounts of the P450, suggesting that the increased accumulation of the substrate in the *tolC acrAB* mutant contributes to enhanced biotransformation.

Several different multidrug resistance efflux systems in *E. coli* have been identified. All of them consist of an outer membrane factor (OMF) and a membrane fusion protein.[8] About OMFs, it was reported that TolC is a

major example in *E. coli*, and that other OMFs (Yjcp, YohG, and YlcB) hardly contribute to efflux pump systems.[8] We demonstrated that *E. coli* strains with *tolC* mutation accumulated substrates and showed more efficient biotransformation efficiency, and that *acrAB* mutation into the *tolC* mutant had additive effects. These additive effects suggest possibilities of AcrAB-couplings with other OMFs and export by these complexes. That is, inactivation of other OMFs-AcrAB efflux pumps could have some additive effects on the accumulation of substrates. Indeed, a previous study proposed the possibility that two putative members of the OMF also affect intrinsic resistance in addition to TolC, based on the fact that deletion of *yjcP* and *yohG* resulted in a 4-fold increase in susceptibility to some limited antibiotics (puromycin, acriflavin, and tetraphenylarsonium chloride of 35 antibiotics).[8] We identified that the vast majority of products oxidized by biotransformations using the *tolC* and/or *acrAB* mutant strains did not localize in intracellular domains but in the extracellular media (data not shown). These results also suggest the existence of TolC-independent efflux systems to export these products. Fortunately, this localization of products in extracellular media prevented the decline of our biotransformation efficiency.

Examples of membrane fusion proteins are AcrAB, EmrAB, AcrEF, and the predicted YhiVU and EmrKY.[8] The decrease intracellular amounts of VD3 and 4C3O in the *acrAB* mutant (*tolC*$^{+}$) strain might have been caused by complementation of the deleted AcrAB with these other membrane fusion proteins. Accordingly, other membrane fusion proteins might be able to couple with TolC in place of AcrAB and to take part in the more effective efflux of VD3 and 4C3O rather than AcrAB. A previous study on the AcrAB-TolC efflux pump revealed that both mutations of *tolC* and *acrAB* became sensitive to various organic solvents to similar extents.[7] However, our data showed that the effects of *acrAB* mutation on substrate accumulation and biotransformation efficiency were much lower than that of *tolC* mutation. AcrAB-independent efflux systems might take part in the efflux of relatively large compounds more than small organic solvents.

Here we demonstrated versatile methods for efficient biotransformations of hydrophobic and amphiphilic compounds to produce *regio*-specific oxidized products using *E. coli* strains with *tolC acrAB* mutations expressing P450 genes. We have realized that our methods probably improve the efficiency of biotransformations using enzymatic genes other than P450s.

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