Biocatalyst Engineering by Assembly of Fatty Acid Transport and Oxidation Activities for In Vivo Application of Cytochrome P-450_{BM-3} Monooxygenase

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The application of whole cells containing cytochrome P-450_{BM-3} monooxygenase [EC 1.14.14.1] for the bioconversion of long-chain saturated fatty acids to ω -1, ω -2, and ω -3 hydroxy fatty acids was investigated. We utilized pentadecanoic acid and studied its conversion to a mixture of 12-, 13-, and 14-hydroxypentadecanoic acids by this monooxygenase. For this purpose, *Escherichia coli* recombinants containing plasmid pCYP102 producing the fatty acid monooxygenase cytochrome P-450_{BM-3} were used. To overcome inefficient uptake of pentadecanoic acid by intact *E. coli* cells, we made use of a cloned fatty acid uptake system from *Pseudomonas oleovorans* which, in contrast to the common FadL fatty acid uptake system of *E. coli*, does not require coupling by FadD (acyl-coenzyme A synthetase) of the imported fatty acid to coenzyme A. This system from *P. oleovorans* is encoded by a gene carried by plasmid pGec47, which has been shown to effect facilitated uptake of oleic acid in *E. coli* W3110 (M. Nieboer, Ph.D. thesis, University of Groningen, Groningen, The Netherlands, 1996). By using a double recombinant of *E. coli* K27, which is a *fadD* mutant and therefore unable to consume substrates or products via the β -oxidation cycle, a twofold increase in productivity was achieved. Applying cytochrome P-450_{BM-3} monooxygenase as a biocatalyst in whole cells does not require the exogenous addition of the costly cofactor NADPH. In combination with the coenzyme A-independent fatty acid uptake system from *P. oleovorans*, cytochrome P-450_{BM-3} recombinants appear to be useful alternatives to the enzymatic approach for the bioconversion of long-chain fatty acids to subterminal hydroxylated fatty acids.

Cytochrome P-450_{BM-3} monooxygenase (CytP450_{BM-3}) is a soluble NADPH-dependent monooxygenase from *Bacillus megaterium* ATCC 14581 (13). It is a class II P-450 enzyme that contains flavin adenine dinucleotide, flavin mononucleotide, and a heme moiety (17). Unlike most CytP450 monooxygenases, which consist of a distinct monooxygenase and a reductase, CytP450_{BM-3} contains these functionalities in a single polypeptide (3, 15, 18).

The enzyme hydroxylates a variety of long-chain aliphatic substrates, such as fatty acids, alkanols, and alkylamides at the ω -1, ω -2, and ω -3 positions (4, 17), and oxidizes unsaturated fatty acids to epoxides in vitro (17, 23) with high enantioselectivity. Oxidation of eicosapentenoic acid (C_{20:5}) and arachidonic acid (C_{20:4}) yielded 17(*S*),18(*R*)-epoxyeicosatetraenoic acid (94% enantiomeric excess [e.e.]) for the former and a mixture of 18-(*R*)-hydroxyarachidonic acid (92% e.e.) and 14(*S*),15(*R*)-epoxyeicosatrienoic acid at 98% e.e. for the latter substrate (8). Recently, it has been demonstrated that the enzyme also produces α,ω diacids from ω -oxo fatty acids by oxidation of the terminal aldehyde functionality (9). The catalytic constant (k_{cat}) of CytP450_{BM-3} is among the highest found for P-450 monooxygenases, ranging from 15 s⁻¹ for laureate to 75 s⁻¹ for pentadecanoic acid (11). For comparison, a typical microsomal P-450 monooxygenase from human liver (CYP2J2) had a k_{cat} of 10⁻³ s⁻¹ for arachidonic acid (32), compared to a k_{cat} of 55 s⁻¹ for CytP450_{BM-3} for the same substrate (8).

This high catalytic efficiency prompted us to investigate the

applicability of CytP450_{BM-3} as a biocatalyst for the subterminal hydroxylation of long-chain fatty acids (LCFAs). Since these subterminal hydroxy LCFAs are chiral molecules, their application in the production of enantiopure synthetic building blocks, especially for pharmaceutical agents, could be envisioned. Further, long-chain hydroxy acids find applications as precursors for polymers or cyclic lactones, which are used as components of fragrances and as antibiotics. Although chemical syntheses have been developed for ω -1 hydroxy fatty acids (from C₁₂ to C₁₈) (26, 28, 29) and for ω -2 and ω -3 hydroxyoctadecanoic acids (2), they require expensive functionalized substrates and are in general complicated, multistep processes (26, 28, 29) which cannot be carried out with unmodified fatty acids as inexpensive starting material. In principle, such inexpensive substrates can be oxidized to hydroxy fatty acids by biocatalysts, either in vitro or in vivo. The latter is preferred, since whole cells actively regenerate the NADPH required for fatty acid oxidation with monooxygenases such as CytP450_{BM-3}. In designing a suitable whole-cell biocatalyst, several additional points had to be considered.

First, uptake must be efficient. Second, degradation of substrate or product must be avoided. In fact, biotransformations of fatty acids with whole cells are usually inefficient due to limited uptake of these compounds at neutral pH, and when taken up, they are degraded via β -oxidation. The transport of LCFAs in *Escherichia coli* is mediated via the *fadL* and *fadD* gene products. FadL is the transporter that carries LCFAs across the outer membrane and is absolutely required for LCFA transport (20). FadD, the acyl coenzyme A (CoA) synthetase, is located at the inner side of the cytoplasmic membrane and is required for formation of the acyl coenzyme A thioester, after which the activated fatty acids are channeled

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference(s)
<i>E. coli</i> strains		
JM101	F' (<i>traD36 lacI^q lacZΔM15 proAB</i>) <i>supE thi-1 Δ(lac-proAB) endA</i> <i>hsdR</i>	33
K27	<i>tyrT58(ΔS) fadD88 mel-1</i>	21
W3110	F ⁻ , λ ⁻ , IN(<i>rrnD-rnE</i>) ₁	1
Plasmids		
pCYP102	pUC13 carrying pCYP102, <i>bla</i>	24, 30
pUC18	<i>lacZα</i> , ColE1 replicon, <i>bla</i>	33
pGEc47	pLAFRI carrying <i>alkBFGHJKL</i> and <i>alkST</i> , <i>tet</i>	10

into the β -oxidation cycle for fatty acid degradation (21, 22). Thus, we used a FadD mutant, *E. coli* K27, as a suitable host for the production of subterminal hydroxyalkanoic acids (20). *E. coli* K27 cannot couple free fatty acids to coenzyme A, thus preventing substrate or product degradation by the host. Such *fadD* mutants are, however, also impaired in efficient uptake of fatty acids (20). We circumvented this by introducing a fatty acid uptake system from *Pseudomonas oleovorans* encoded on pGEc47. Finally, we introduced the P-450_{BM-3} monooxygenase on pCYP102 into the *fadD* mutant *E. coli*. The resulting recombinant, *E. coli* K27(pCYP102, pGEc47), is a promising tailored biocatalyst for the oxidation of saturated LCFAs to ω -1, ω -2, and ω -3 hydroxy fatty acids.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids that were used in this study are listed in Table 1. Strains were transformed with plasmid pCYP102 encoding CytP450_{BM-3} (24, 30) and vector without insert (pUC18) as a control. The effect of enhanced fatty acid uptake on CytP450_{BM-3}-mediated oxidation was studied in *E. coli* double recombinants. *E. coli* JM101 (33), W3110 (1), and K27 (21) were used as host microorganisms. *E. coli* K27, a *fadD* mutant, is blocked in fatty acid degradation since it lacks acyl CoA synthetase activity (21) and is thus unable to consume CytP450_{BM-3} substrates or products.

Chemicals. Pentadecanoic acid, nonanoic acid, and solvents for gas chromatography (GC) and mass spectrometry (MS) analysis such as pyridine and *N,O*-bis-(trimethylsilyl)-trifluoroacetamide were obtained from Sigma (Buchs, Switzerland). Salts for buffer solutions and dimethylsulfoxide (DMSO) were purchased from Fluka (Buchs, Switzerland). Diazomethane was purchased from Hoffmann-La Roche AG (Basel, Switzerland). NADPH and dithiothreitol (DTT) were obtained from Gerbu (Gaiberg, Germany). Ampicillin and tetracycline were from Boehringer (Mannheim, Germany). Pefablock protease inhibitor was obtained from Merck (Darmstadt, Germany).

Media and growth conditions. All cultivations were carried out at 37°C. Shaking flask experiments were performed in 250-ml Erlenmeyer flasks filled with 50

FIG. 1. Expression of CytP450_{BM-3} and AlkB in *E. coli* JM101(pCYP102, pGEc47), *E. coli* K27(pCYP102, pGEc47), and *E. coli* W3110(pCYP102, pGEc47) in the absence and presence of DCPK. Samples were grown in M9 medium with glucose as the C source (0.5% glucose [wt/vol]) and were harvested at late-exponential phase. Whole-cell extracts were prepared, and 20 μ g of protein of each sample was analyzed on an SDS-8% polyacrylamide gel. Lanes 1 and 5, *E. coli* JM101(pCYP102, pGEc47); lanes 2 and 6, *E. coli* K27(pCYP102, pGEc47); lanes 3 and 7, *E. coli* W3110(pCYP102, pGEc47). Lanes 1 through 3 show cultures grown in the absence of the *alk* inducer DCPK. Lane 4, purified *alkB*. Lanes 5 through 7 show proteins of the recombinants grown in the presence of DCPK (0.05% [vol/vol]). The molecular mass standard is shown in lane 8. The arrow indicates the AlkB band at 42 kDa. The asterisks identify the P-450_{BM-3} band at 119 kDa.

ml of medium. Mineral M9 medium (25) was prepared with 0.5% (wt/vol) glucose, MgSO₄ (5 mM), CaCl₂ (0.1 mM), and 1% thiamine (except for W3110). The recombinants were precultured in Luria-Bertani medium and transferred to M9 medium to an initial density of approximately 0.05 mg/ml based on the absorbance at 450 nm (31). Tetracycline (12.5 μ g/ml) and/or ampicillin (150 μ g/ml) was used when required.

Oxidation of saturated LCFAs by resting cells of CytP450_{BM-3}-producing recombinants. For bioconversion studies with resting cells, we grew *E. coli* K27, W3110, or JM101 cells to late-exponential phase (0.8 g liter⁻¹ cell dry weight [cdw]) in M9 minimal medium with glucose (0.5%, wt/vol) as the carbon source. Cells were resuspended to a density of 2 g liter⁻¹ cdw in 0.2 M potassium phosphate buffer (pH 7.4) containing 0.5% (wt/vol) of the carbon source. The cultures were incubated at 37°C in airtight baffled Erlenmeyer flasks on a rotary shaker at 250 rpm after the addition of 5 mM pentadecanoic acid (from a 100 mM stock solution in DMSO). To reduce the occurrence of multiple oxidations of the substrates leading to, e.g., ketones, as side products (6), some conversions were performed under oxygen-limited conditions by sparging a closed flask with nitrogen. Samples of 1 ml were taken through the top of the bottle at defined stages of incubation and were prepared immediately for GC analysis. To compare the rates of oxidation of different LCFAs (ranging from C₁₂ to C₁₈) by *E. coli* K27(pCYP102, pGEc47), a batch culture of growing cells was split into seven equal amounts under sterile conditions. The aliquots were supplemented with 5 mM LCFAs (dissolved in DMSO) of different chain lengths and were incubated under oxygen-limited conditions. After 4 h of conversion, a 1-ml sample of each subculture was taken for GC-MS analysis.

TABLE 2. Localization of pentadecanoic acid and hydroxy products from whole-cell bioconversion of C_{15:0} by different *E. coli* recombinants^a

<i>E. coli</i> strain	Plasmid			% C _{15:0} in ^b :		% Products in:		% of substrate + products in:	
	pCYP102	pGEc47	pUC18	Supernatant	Cell pellet	Supernatant	Cell pellet	Supernatant	Cell pellet
K27	+	+	—	12	54	10	2	22	56
	+	—	—	42	38	4	2	46	40
	—	+	+	14	58			14	58
	—	—	+	46	22			46	22
JM101	—	+	+	18	12			18	12
	—	—	+	30	24			30	24
W3110	—	+	+	20	34			20	34
	—	—	+	22	36			22	36

^a Determinations of fatty acid content by GC analysis were based on a 4-h conversion and repeated three times. +, plasmid; —, no plasmid.

^b Percentage of the original (5 mM) fatty acid amount in the supernatant or cell pellet.

TABLE 3. Bioconversion of pentadecanoic acid by *E. coli* recombinants carrying different plasmids^a

<i>E. coli</i> strain	Plasmid ^b			Rate of production ^c (U g ⁻¹ cdw)
	pCYP102	pGEc47	pUC18	
K27	+	+	—	1.3 ± 0.15
	+	—	—	0.7 ± 0.1
	—	+	+	—
	—	—	+	—
JM101	+	+	—	0.53 ± 0.1
	+	—	—	0.25 ± 0.07
	—	+	+	—
	—	—	+	—
W3110	+	+	—	0.46 ± 0.1
	+	—	—	0.16 ± 0.07
	—	+	+	—
	—	—	+	—

^a Rates of production are based on a 4-h conversion under oxygen-limited conditions; determinations were repeated three times.

^b +, plasmid; —, no plasmid.

^c —, no activity.

Preparation of cell-free extracts. A culture of recombinant *E. coli* K27(pCYP102, pGEc47) was grown to late-exponential phase. Cells were harvested by centrifugation (5,000 × g, 4°C, 30 min) and resuspended in 0.2 M potassium phosphate buffer (pH 7.4) containing 2 mM DTT and 2 mM pefablock. Cells were disrupted in a French press (SLM Instruments, Urbana, Ill.) and were centrifuged (5,000 × g, 4°C, 30 min) to remove intact cells and debris. The supernatant was used for in vitro oxidation assays.

Oxidation of saturated LCFA with cell-free extracts containing CytP450_{BM-3}. Cell-free extracts were incubated under oxygen-limited conditions (as described above) at 37°C in Erlenmeyer flasks on a rotary shaker at 250 rpm after the addition of 5 mM pentadecanoic acid from a 100 mM stock solution in DMSO and 5 mM NADPH (from a 100 mM stock solution in potassium phosphate buffer). Cultures that were used to compare bioconversions by whole cells or extracts from *E. coli* K27(pCYP102, pGEc47) originated from one starter culture, which was split in two.

SDS-PAGE and protein content. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (12). Samples containing 20 µg of protein were loaded on an 8% polyacrylamide gel. As a molecular marker reference, we used commercially available broad-range protein markers from Bio-Rad (6.5 to 200 kDa). Protein bands were visualized by staining with Coomassie brilliant blue.

Protein concentrations were determined with the Bio-Rad protein assay based on the Bradford dye-binding procedure (7).

Localization and determination of pentadecanoic acid content from bioconversions by different *E. coli* recombinants. Resting cells (2 g liter⁻¹ cdw) of *E. coli* recombinants were incubated for 4 h in the presence of 5 mM C_{15:0} and glucose (0.5%, wt/vol) at 37°C and 250 rpm. Samples of 1 ml were taken, centrifuged, and separated into cell pellet and supernatant. The pellet was washed three times with phosphate buffer (pH 7.4), and the supernatant and pellet fraction were used for pentadecanoic acid determination by GC analysis.

Gas chromatography of fatty acid metabolites. Prior to GC and GC-MS analysis, 1-ml samples were acidified by HCl (pH 2) and extracted with hexane, and methyl esters were generated from the carboxylic acids by adding 20 µl of diazomethane. The derivatized hexane extract was dried over sodium sulfate, and 1 µl of this solution was analyzed on a capillary gas chromatograph (HRGC MEGA2 series; Fisons Instruments) with a 25-m CP-Sil 5CB column (Chrompack, Middelburg, The Netherlands). The temperature program used was 80°C for 2 min, a temperature gradient of 8°C/min to 240°C, and isothermic at 240°C for 10 min. In some cases, to achieve better product separation a less-steep temperature program was chosen: 120°C for 1 min, a temperature gradient of 1°C/min to 240°C, and isothermic at 240°C for 10 min. Nonanoic acid (3.5 mM) from a 100 mM stock solution in DMSO was added to the assay mixture before acidification of the sample to serve as an internal standard. Quantification by GC of the hydroxylation products was achieved by comparison of the signal intensity with that of the internal standard and corrected for differences in flame response (22). Rates were calculated from the amount of products formed per unit of time and cell dry weight or protein content.

Identification of the oxidation products by GC-MS. For GC-MS analysis, CytP450_{BM-3} oxidation products were derivatized as described above and the GC column and separation conditions were kept identical. A Fisons GC800 gas chromatograph coupled to a Fisons MD800 mass selective detector was used (H₂ carrier gas; flow, 1 ml/min; electron impact energy, 70 eV). Authentic standards

FIG. 2. Bioconversion of C_{15:0} by *E. coli* K27(pCYP102, pGEc47) and *E. coli* K27(pCYP102). Resting cells harvested at late-exponential phase and cell-free extracts (2 g liter⁻¹ cdw) resuspended from M9 minimal medium (0.5% glucose [wt/vol]) were incubated with 5 mM pentadecanoic acid at 37°C and 250 rpm. (A) Substrate depletion and product formation were monitored over a 10-h biotransformation by intact cells of *E. coli* K27(pCYP102, pGEc47) (squares) and *E. coli* K27(pCYP102) (circles), synthesizing CytP450_{BM-3}. (B) Substrate depletion and product formation over a 10-h conversion of pentadecanoic acid by cell-free extracts of *E. coli* K27(pCYP102, pGEc47) containing 5 mM NADPH. Product amounts were calculated as the sums of 12-, 13-, and 14-hydroxypentadecanoic acids (identified by GC-MS analysis). S, substrates (open symbols); P, products (filled symbols).

for the products are not available, and fragment distribution was therefore used to determine the absolute configuration of the compounds produced by the bioconversion reaction. Product characterization was performed based on the expected cleavage pattern of hydroxy-methyl ester derivatives. The procedure enabled us to distinguish between the ω -1, ω -2, and ω -3 hydroxy fatty acids, since electron impact ionization usually results in cleavage of *sec* alcohols adjacent to the hydroxyl moiety (14).

Detailed GC-MS data for all of the ω -1, ω -2, and ω -3 hydroxy fatty acids are available as supplementary material from the corresponding author upon request.

RESULTS

Growth and *cyp102* gene expression of *E. coli* recombinants carrying pCYP102 and pGEc47. Fatty acids were oxidized to subterminal hydroxy fatty acids by recombinants of *E. coli* JM101, W3110, or K27 containing plasmid pCYP102. Control experiments to verify that oxidation was CytP450_{BM-3} dependent were done with strains carrying pUC18 instead of pCYP102. To demonstrate the effect of fatty acid uptake encoded by the OCT plasmid genes, pGEc47 was included in

FIG. 3. GC-MS chromatogram (total ion current [TIC]) of fatty acid C₁₂ to C₁₈ oxidation products after bioconversion by *E. coli* K27(pCYP102, pGEc47) under low-oxygen conditions. Cells were grown as described in Materials and Methods. Seven aliquots were supplemented with 5 mM C₁₂ to C₁₈ saturated fatty acids (dissolved in DMSO) and incubated at 37°C under conditions of limited oxygen. After 4 h, 1 ml of each aliquot was taken for GC-MS analysis. The panels show the GC profiles obtained after oxidation of the fatty acids indicated. The broad peak in each panel represents unoxidized substrate; the hydroxy fatty acid products are shown at the right, marked by their retention times. For separation of these peaks, see Fig. 4.

these recombinants and these strains were compared to negative controls without the plasmid.

The various recombinants were cultivated at 37°C in M9 minimal medium containing 0.5% (wt/vol) glucose, glycerol, lactose, succinate, or pyruvate as the carbon source. Comparing the growth behaviors of transformants of single and double recombinants of *E. coli* JM101, W3110, and K27 and their levels of expression of CytP450_{BM-3} demonstrated that glucose was the optimal C source.

The presence of pGEc47 and pCYP102 gene products in the recombinants was tested by SDS-PAGE (Fig. 1). In the presence of the *alk* inducer dicyclopropylketone (DCPK), the AlkB protein (42 kDa) was prominently present. Similarly, plasmid pCYP102 expressed CytP450_{BM-3} as a 119-kDa band (Fig. 1). Since the involvement of pGEc47 in the transport of fatty acids was previously demonstrated only in *E. coli* W3110 (19), this strain was included in our experiments. However, while AlkB

was expressed efficiently in *E. coli* W3110(pCYP102, pGEc47), CytP450_{BM-3} was not (Fig. 1). The best recombinants based on lysate analysis were *E. coli* K27(pCYP102, pGEc47) and *E. coli* JM101(pCYP102, pGEc47).

Involvement of pGEc47 in fatty acid uptake. To test the influence of pGEc47 on facilitated substrate uptake, recombinant *E. coli* strains were grown for 4 h in the presence of 5 mM pentadecanoic acid. The cells were then harvested and washed, and the C₁₅ content of the cell pellet and supernatant was determined by GC analysis (Table 2). The JM101 and W3110 recombinants consumed more of the supplied fatty acid than did the K27 recombinants. That which remained was found to a greater extent in the supernatant of JM101 and intracellularly in W3110. For recombinants carrying pGEc47, most fatty acid was found in the cell pellet and only 0.6 to 1.0 mM substrate was detected in the supernatant. As expected, K27 recombinants consumed very little of the supplied fatty acid,

and the recombinants carrying pGEc47 accumulated more than half of the total fatty acids intracellularly. In contrast, concentrations of 2.1 mM and 2.3 mM pentadecanoic acid remained in the supernatant of cultures of *E. coli* K27(pCYP102) and *E. coli* K27(pUC18) recombinants that were not equipped with pGEc47.

Fatty acid degradation by *E. coli* recombinants. The amounts of unoxidized free pentadecanoic acid and the formation of 12-, 13-, and 14-hydroxypentadecanoic acids by the different *E. coli* recombinants were quantified (Table 2). *E. coli* K27 recombinants demonstrated higher molecular recovery than the *fadD*⁺ strains after a 4-h period. Recombinants of *E. coli* JM101 and *E. coli* W3110 had lower overall recoveries: between 40 and 70% of the added substrate was consumed by these *fadD*⁺ strains. The overall recovery of substrate plus products for the *fadD* recombinants varied from 70 to 90%.

Bioconversion of pentadecanoic acid to hydroxypentadecanoic acids. Oxidation of pentadecanoic acid by CytP450_{BM-3} under low-oxygen conditions has been shown to reduce the formation of multiple oxidation products, such as ketopentadecanoic acids, keto-hydroxypentadecanoic acids, and dihydroxypentadecanoic acids, using purified enzyme (6). We tested the pentadecanoic acid oxidation activity under low-oxygen conditions of various *E. coli* recombinants harboring pCYP102, pGEc47, and pUC18 in different combinations. With all *E. coli* recombinants harboring the pCYP102 gene, formation of the three ω -1, ω -2, and ω -3 hydroxypentadecanoic acid products was observed by GC-MS analysis. The best activity (1.3 U g⁻¹ cdw) was seen for *E. coli* K27(pCYP102, pGEc47) carrying the *fadD* mutation (Table 3). *E. coli* K27(pCYP102) converted pentadecanoic acid to the three hydroxy products at a maximum rate of 0.7 U g⁻¹ cdw, while *E. coli* W3110(pCYP102) had an activity of only 0.16 U g⁻¹ cdw, in accordance with the low level of CytP450_{BM-3} seen in this recombinant (Fig. 1).

We also monitored the hydroxylation of 5 mM pentadecanoic acid over a longer period of time in cultures growing in M9 defined medium supplied with 0.5% glucose as a carbon source (Fig. 2A). Although the initial rate of product synthesis was about 5 U g⁻¹ cdw, it decreased and reached an almost constant value of 1 U g⁻¹ cdw after about 6 h for *E. coli* K27(pCYP102, pGEc47). This resulted in the synthesis of hydroxypentadecanoic acids to a final concentration of 1.2 mM (0.3 g liter⁻¹) after 10 h of conversion. In contrast, *E. coli* K27(pCYP102) produced subterminal hydroxylated fatty acids to a concentration of 0.46 mM (0.11 g liter⁻¹) after the same conversion time, which corresponds to an overall activity of 0.4 U g⁻¹ cdw (Fig. 2A). The conversion was continued, and after 40 h, we found subterminal hydroxylated fatty acids at total concentrations of 1.85 mM by using *E. coli* K27(pCYP102, pGEc47) and of 0.65 mM by using *E. coli* K27(pCYP102) cultures. No product formation was observed for recombinants carrying pUC18 or pUC18 and pGEc47.

Oxidation of pentadecanoic acid by CytP450_{BM-3} in cell-free extracts versus whole cells. Cell-free extracts of *E. coli* K27(pCYP102, pGEc47) expressing CytP450_{BM-3} efficiently produced 12-, 13-, and 14-hydroxypentadecanoic acids from 5 mM pentadecanoic acid in the presence of 5 mM NADPH. Thus, the overall productivity of extracts was about threefold higher than that of intact cells of the same recombinant (Fig. 2B). Whereas intact cells of *E. coli* K27(pCYP102, pGEc47) produced hydroxylated fatty acids to a concentration of 1.85 mM after 40 h, cell-free extracts of the same culture produced this amount in 2 h. The activity decreased after 30 min, probably due to NADPH depletion, but eventually resulted in formation of 2.3 mM hydroxy fatty acids after 5 h. Repeated

FIG. 4. Hydroxy fatty acid isomer distribution. GC-MS chromatograms of C₁₂ through C₁₈ hydroxy fatty acid products show the distribution among the ω -1, ω -2, and ω -3 isomers. A less-steep gradient of the GC program resulted in separation of the single hydroxylated products.

experiments showed that the use of cell-free extracts yielded higher enzyme activities of up to 30 U g of total protein⁻¹ (based on a 30-min conversion time). After the same conversion time, whole cells of *E. coli* K27(pCYP102, pGEc47) produced 0.3 mM hydroxypentadecanoic acids with a productivity of 5 U g⁻¹ cdw.

Oxidation of C₁₂ to C₁₈ LCFAs by *E. coli* K27(pCYP102, pGEc47). Saturated LCFAs, which are substrates of purified CytP450_{BM-3} monooxygenase (5), were oxidized in whole-cell bioconversions with *E. coli* K27(pCYP102, pGEc47) under low-oxygen conditions. As shown in Fig. 3, there was significant oxidation of all fatty acids with chain lengths ranging from C₁₂ to C₁₈ after 4 h of incubation. By GC-MS analysis, we could identify ω -1, ω -2, and ω -3 hydroxy fatty acids as conversion products (Fig. 4). The rates of formation of the individual hydroxy products, calculated after a 4-h incubation period, are shown in Table 4. Similar to the relative substrate conversion rates with the purified enzyme (16), we found that the whole-cell biocatalyst oxidized C_{14:0} and C_{15:0} most efficiently (1.67

TABLE 4. Conversion rates of C_{12:0} to C_{18:0} fatty acids to ω -1, ω -2, and ω -3 hydroxy fatty acid products by *E. coli* K27(pCYP102, pGEc47)^a

Substrate fatty acid	Hydroxy fatty acid product formation (U g ⁻¹ cdw)			Total product formation (Ug ⁻¹ cdw)
	ω -1 ^b	ω -2 ^b	ω -3 ^b	
C _{12:0}	0.25 ± 0.04	≤0.17	0.21 ± 0.03	0.63 ± 0.07
C _{13:0}	≤0.08	0.63 ± 0.07	≤0.13	0.84 ± 0.07
C _{14:0}	0.83 ± 0.1	0.42 ± 0.05	0.42 ± 0.05	1.67 ± 0.2
C _{15:0}	0.42 ± 0.05	0.63 ± 0.07	0.31 ± 0.04	1.36 ± 0.12
C _{16:0}	≤0.13	≤0.19	≤0.10	≤0.42
C _{17:0}	≤0.07	≤0.08	≤0.05	≤0.20
C _{18:0}	≤0.03	≤0.04	≤0.03	≤0.10

^a Rates of product formation calculated by GC analysis are based on a 4-h fatty acid conversion and three determinations.

^b Position of the hydroxy group in the hydroxy fatty acid.

and 1.36 U g⁻¹ cdw, respectively). Octadecanoic acid was transformed least efficiently (0.1 U g⁻¹ cdw; sum of three oxidation products). As illustrated in Fig. 4, the distribution of hydroxylated regioisomers is not consistent for all fatty acids. Some fatty acids (e.g., C_{12:0} and C_{14:0}) were preferentially oxidized at the ω -1 position, whereas C_{13:0}, C_{15:0}, C_{16:0}, C_{17:0}, and C_{18:0} were most efficiently oxidized at ω -2 (Table 4).

DISCUSSION

E. coli K27(pCYP102, pGEc47) versus *E. coli* K27(pCYP102) as catalyst for the bioconversion of pentadecanoic acid. Under reduced-oxygen conditions, the whole-cell biotransformation of pentadecanoic acid carried out by *E. coli* K27(pCYP102, pGEc47) and *E. coli* K27(pCYP102) resulted in the formation of 12-, 13-, and 14-hydroxypentadecanoic acids only. Most of these products were detected primarily in the cell supernatant, which indicates that the hydroxylated compounds were excreted by the cells.

Our results showed that the uptake of pentadecanoic acid by the hosts studied was stimulated by the presence of pGEc47. An uncharacterized fatty acid uptake system has been associated with a fragment of pGEc47 which contains DNA from the OCT plasmid of *P. oleovorans* (10). This plasmid was shown to be involved in the efficient uptake of oleic acid by *E. coli* W3110 (19), and in conjunction with this activity an open reading frame encoding a putative cytoplasmic membrane transporter has been identified (27). In our studies, recombinants equipped with pGEc47 demonstrated improved transport of exogenous pentadecanoic acid to the cell interior (Table 2), which enhanced the rate of pentadecanoic acid oxidation to the ω -1, ω -2, and ω -3 hydroxy isomers by up to twofold (Table 3).

We also found that *fadD* mutants proved useful to prevent degradation of the substrate and the desired products via β -oxidation (Table 2). Recombinants of *E. coli* K27 showed higher molecular recoveries than did those of the other strains, which are *fadD*⁺, indicating reduced degradation of the offered fatty acids by *E. coli* K27 recombinants.

Thus far, the application of CytP450_{BM-3} as a biocatalyst has been limited due to its strict requirement of stoichiometrically added NADPH in enzyme-based reactions. Whole cells, used for the bioconversion of pentadecanoic acid, were approximately 30% as active as the corresponding cell-free extracts of *E. coli* K27(pCYP102, pGEc47) in producing 12-, 13-, and 14-hydroxypentadecanoic acids. Conversion by whole cells may proceed more slowly due to mass transfer limitation of the

charged substrates across the cell wall. We believe that the observed differences (Fig. 2) are primarily due to the fact that in the in vivo system intracellular oxidation by CytP450_{BM-3} is limited by the available pool of NADPH, which must be regenerated by cellular metabolism in whole-cell bioconversions.

Nevertheless, whole cells are preferred to cell-free extracts for the oxidation of LCFAs with CytP450_{BM-3}, since in vitro NADPH regeneration can be avoided. By equipping the *fadD*-deficient recombinant *E. coli* K27(pCYP102) with pGEc47, the application of CytP450_{BM-3} as part of a whole cell biocatalyst appears feasible. The availability of sufficient quantities of CytP450_{BM-3} in a whole-cell biocatalytic system opens the way to the practical oxidation of LCFAs to hydroxy LCFAs and unsaturated LCFAs to optically active epoxides.

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