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Development of a Novel *Escherichia coli* Host Strain for Long-chain Fatty Acid Hydroxylation

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긴사슬지방산의 수산화반응을 위한 새로운 대장균 숙주 개발

지도교수 김병기

이 논문을 공학석사 학위논문으로 제출함

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배 진 형의 공학석사 학위논문을 인준함

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Abstract

Hydroxy long-chain fatty acids, having both a carboxyl group and a hydroxyl group, are precursors for pseudo-ceramides, polyesters, and lactones. A major problem of hydroxylation of long-chain fatty acid (LCFA) in *Escherichia coli* is that transport and degradation of LCFA are tightly linked by long-chain fatty acyl-CoA synthetase (FadD). In this study, an effective way to prevent degradation of fatty acid without impairing transport was proposed. It requires manipulating two endogenous proteins related with LCFA transport, i.e. FadD and FadL, and a heterologous enzyme which consumes LCFA. CYP153A from *Marinobacter aquaeolei* which converts palmitic acid into ω-hydroxy palmitic acid was expressed in *E. coli* as a model system. With CYP153A in the cell, the ability to transport LCFA was successfully maintained even when *fadD* was deleted. However, the lack of long-chain fatty acyl-CoA (LCFA-CoA), which is synthesized by FadD, is known to downregulate outer membrane LCFA transporter gene (*fadL*). This problem was solved by the overexpression of *fadL* from an additional vector. It relieved not only repression by FadR but also catabolite repression to allow glucose to be used as carbon source which can be rapidly catabolized. When *fadD* deletion and *fadL* overexpression were combined, 2.6 g/L of palmitic acid was converted to 2.4 g/L of ω-hydroxy palmitic acid,

which was 5.5-fold increase compared to wild-type strain. This simple genetic manipulation generally can be applied to any LCFA hydroxylation using *E. coli.*

Key words: Hydroxy long-chain fatty acid, Long-chain fatty acid hydroxylation, Long-chain fatty acid degradation, Long-chain fatty acid transport, FadD, FadL

Contents

List of Figures

List of Tables

Abbreviations

LCFA: Long-chain fatty acid

ALA: 5-Aminolevulinic acid

BSTFA: N,O-Bis(trimethylsilyl)-trifluoroacetamide

DMSO: Dimethyl sulfoxide

LB: Luria-Bertani

TB: Terrific broth

TMS: Trimethylsilyl

DCW: Dry cell weight

BLAST: Basic local alignment search tool

1. Introduction

Hydroxy long-chain fatty acids, having both a carboxyl group and a hydroxyl group, are precursors for pseudo-ceramides, polyesters, and lactones. Although chemical hydroxylation of long-chain fatty acid (LCFA) is possible by metathesis (1) or using transition metal as the catalyst (2), often harsh reaction condition or regioselectivity becomes the limitation (3). Enzymes which are able to solve the problem in the synthesis of hydroxy LCFA can be found from the nature. Cytochrome P450s, for example, are well-known for their ability to break a C-H σ bond of fatty acid alkyl chain and attach a hydroxyl group to the carbon (4-6). Hydratases break a C-C π bond in the middle of the alkyl chain and attach a hydroxyl group at one of the carbon (7). Laccases turn unsaturated fatty acid into epoxy or hydroxy fatty acid (8).

To exploit hydroxylation activity with such enzymes, *Escherichia coli* is an adequate host because of its rapid growth, well-known physiology, and easy genetic manipulation (9). Especially, its LCFA transport mechanism has been clarified (Fig. 1). In fact, *E. coli* FadL,

an outer membrane LCFA transporter, is the only protein that has been clearly demonstrated to be the free fatty acid transporter until now (10). It transports exogenous LCFA into the periplasm by lateral diffusion (11, 12). The mechanism how LCFA moves through the inner membrane with the aid of FadD also has been proposed (13). FadD, a long-chain fatty acyl-CoA (LCFA-CoA) synthetase, supposedly binds to the inner membrane when there is a signal from a LCFA molecule and abstracts the molecule to synthesize LCFA-CoA (14). This biosynthesis is termed “vectorial acylation” because it enables unidirectional LCFA transport across the inner membrane (15). Deletion of *fadD* resulted in the loss of ability to transport LCFA (16), and indispensable role of FadD in LCFA transport was also confirmed by *in vitro* demonstration (17).

The dilemma of LCFA hydroxylation using *E. coli* is that side pathways including β-oxidation start from LCFA-CoA synthesized during the transport (16, 18). Moreover, hydroxy LCFA is also subjected to β-oxidation when FadD is present (19). The fact that the side reactions cannot be blocked has been the limitation of *E. coli* LCFA hydroxylation (20). There were some alternative methods for

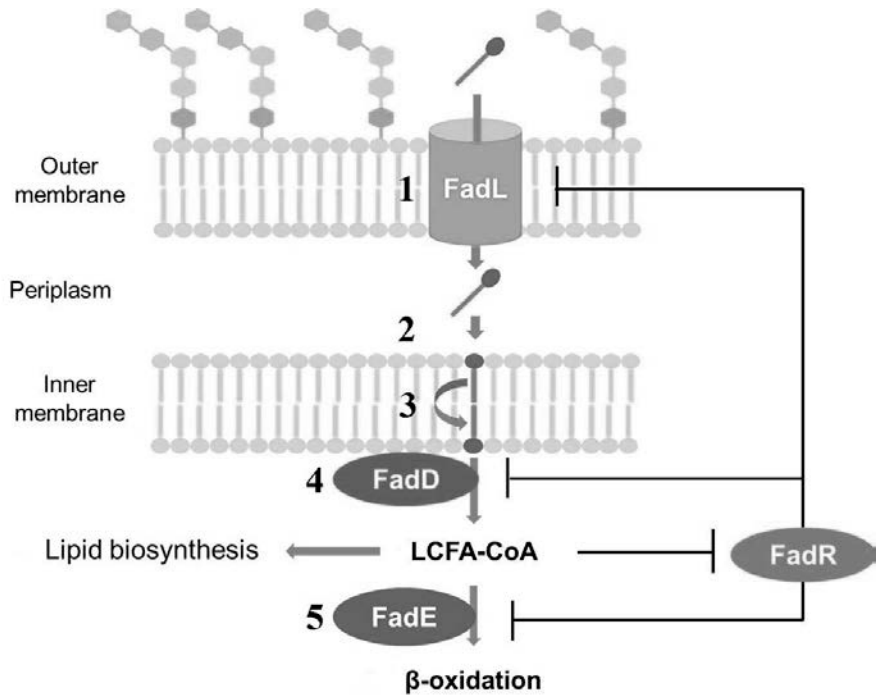

Figure 1. LCFA transport mechanism across *E. coli* membrane. 1) LCFA passes the outer membrane through FadL. 2) LCFA adsorbs to periplasmic side of the inner membrane. 3) LCFA moves to cytosolic side of the inner membrane by flip-flop mechanism. 4) FadD abstracts LCFA to synthesize fatty acl-CoA which can derepress *fad* regulon including *fadL*, *fadD*, and *fadE*. It is also used in lipid biosynthesis. 5) Dehydrogenation by FadE makes fatty enoyl-CoA., the first compound in β-oxidation pathway.

LCFA hydroxylation, such as biosynthesizing LCFA inside the cell with *fadD* deletion (21), adopting heterologous transport system (20), or deleting *fadE* (22) which catalyzes the first step of β-oxidation (23). These approaches, however, are either too much burden to a cell or not capable of preventing side pathways completely which ended up in low productivity. Simple but effective method to construct a host strain with no LCFA side pathway has been sought.

In this study, we developed a method to prevent LCFA side pathways while maintaining the ability to efficiently transport exogenous LCFA in *E. coli*. As the model system, cytochrome P450 reaction system using CYP153A from *Marinobacter aquaeolei* with CamA and CamB from *Pseudomonas putida* as the electron transfer partners which produce ω-hydroxy palmitic acid (24) was chosen (Fig. 2).

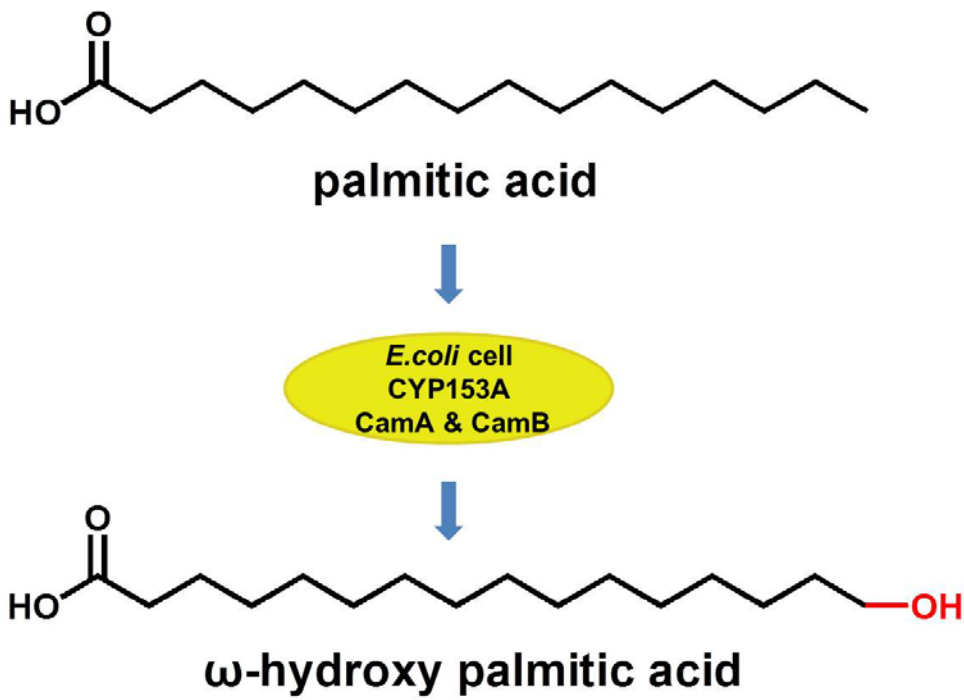

Figure 2. Schematic diagram of ω-hydroxylation of palmitic acid using *E. coli* whole cell reaction.

2. Materials and Methods

1. Chemicals and Media

Palmitic acid (99%), heptadecanoic acid (98%), ω-hydroxy palmitic acid (98%), Triton X-100, 5-aminolevulinic acid (ALA)(98%), and $FeSO_4$ (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)(98%) was obtained from Fluka (Buchs, Switzerland), and chloroform (99%) was from Junsei (Japan). Dimethyl sulfoxide (DMSO) (99%) was purchased from Duksan (Ansan, Korea). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Biosesang (Sungnam, Korea). LB (Luria-Bertani) medium and TB (Terrific broth) medium were obtained from Difco (Sparks, MD, USA).

2. Plasmids and Strains

For expression vectors, pET24-ma(+) (Pasteur Institute, Paris, France), pETDuet-1(Novagen, Madison, Wisconsin, USA), pCDFDuet-1(Novagen), and pCDFmT7 were used. The difference between

pCDFmT7 and pCDFDuet-1 is that pCDFmT7 was modified from pCDFDuet-1 to have only one promoter. Plasmids and strains used in this study are listed in table 1.

Genomic DNA of *Marinobacter aquaeolei* VT8 DSM 11845(ATCC 700491) and *Escherichia coli* BL21 (DE3) were extracted by Exgene Cell SV kit (GeneAll, Seoul, Korea). To construct pP450, *CYP153A* (Maqu_0600) from *M. aquaeolei* gDNA was amplified by PCR with oligonucleotides 5'-ATT ACA TAT GCC AAC ACT GCC CAG AAC-3' and 5'-ATT ACT CGA GAC TGT TCG GTG TCA GTT TG-3', and the gene was inserted into NdeI and XhoI cloning sites of pET-24ma(+). Construction of p*fadL* was done by PCR with oligonucleotides 5'- ATT ACC ATG GTC ATG AGC CAG AAA ACC-3' and 5'-ATT AGA GCT CTC AGA ACG CGT AGT TAA AG-3' which amplified *fadL* (ECD_02268) from *E. coli* gDNA, and the gene was inserted into NcoI and SacI cloning sites of pCDFmT7. For the construction of pCFL, each gene was cut from pP450 or p*fadL* by restriction endonucleases mentioned above and inserted into the corresponding sites. Single-gene knockout mutants were constructed

Table 1. Plasmids and strains used in this study

Name	Description	Reference
Plasmids		
pET24-ma(+)	p15A ori lacI T7 lac Kan^r	(25)
pETDuet-1	pBR322 ori lacI T7 lac Amp^r	Novagen
pCDFDuet-1	CDF ori lacI T7 lac Str^r	Novagen
pCDFmT7	Modified pCDFDuet-1 to harbor one promoter	This study
pP450	pET24-ma(+) encoding for *CYP153A*	This study
pCamAB	pETDuet-1 encoding for *camA* and *camB*	(26)
pFadL	pCDFmT7 encoding for *fadL*	This study
pPFL	pCDFDuet-1 encoding for *CYP153A* and *fadL*	This study
***E. coli* strains**		
BW25113(DE3)	*rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1 λ(DE3)*	(27)
SD	BW25113(DE3) Δ*fadD*	(28)
SE	BW25113(DE3) Δ*fadE*	(28)
SL	BW25113(DE3) Δ*fadL*	(28)
SW1	BW25113(DE3) carrying pP450 and pCamAB	This study

SD1	SD carrying pP450 and pCamAB	This study
SE1	SE carrying pP450 and pCamAB	This study
SL1	SL carrying pP450 and pCamAB	This study
SD2	SD carrying pP450, pCamAB, and pCDFmT7	This study
SD3	SD carrying pP450, pCamAB, and pFadL	This study
SD4	SD carrying pCFL and pCamAB	This study

by the method described by Datsenko *et al* (27).

3. Growth Condition

A fresh colony from an agar plate was selected and incubated in 2 mL of LB medium for 9 hours. 500 Microliters of the culture was then inoculated into 50 mL of LB for resting cell reaction and TB medium for fed-batch reaction. Working concentration of kanamycin, ampicillin, and streptomycin were 50 μg/mL, 100 μg/mL, and 50 μg/mL, respectively. After reaching OD_{600} 0.6~0.7 for LB medium and 0.8~0.9 for TB medium, the culture was pre-incubated at 30 ℃ for 5 mins, and then induction was done by adding 0.01 mM IPTG, 0.5 mM ALA, and 0.1 mM $FeSO_4$. It was then cultured at 30 ℃ at 200 rpm for 12 hours for the expression. When catabolite repression was needed to be induced, 1% (w/v) glucose was supplied after 6 hours of the expression whereas 1% (v/v) glycerol was supplied as control. It was cultured for 6 more hours and prepared for the resting cell reaction as described above. Activity and concentration of P450 of each culture were measured by the method described by Omura *et al*

(29).

4. Resting Cell Reaction

Cells were harvested by centrifugation and washed with ice-cold phosphate buffered saline, followed by resuspension in 100 mM potassium phosphate buffer (pH 7.5) with 1% (w/v) glucose. The resulting cell density was *ca.* 3 g/L. Ten milliliters of the buffer was put in a 100 mL shake flask, and pre-incubated at 30 ℃ at 250 rpm for 10 minutes. Resting cell reaction was initiated by adding 1 mM palmitic acid from 100 mM stock in DMSO.

5. Fed-batch Reaction

Triton X-100 0.1% (v/v) was used as surfactant after 12 hours of protein expression, and pH was adjusted to 7.5 by 5M NaOH. Adding 15 mM palmitic acid (500 mM stock) to 10 mL of the culture in a 100 mL flask initiated the reaction. Every 2 hour, pH was measured by a pH meter. If pH was under 7.0, glucose 0.3 % (w/v) was injected, 0.4% for pH 7.0~7.2, 0.5% for pH 7.2~7.5, and 0.6% for pH 7.5 or above.

6. Measuring Secretion of Hydroxy LCFA

The activity of FadL on hydroxy LCFA secretion was measured by expressing CYP153A, CamA, and CamB in *fadL* deletion mutant. Cells harboring three genes were prepared as described in "growth condition" section. In order to minimize β-oxidation, 1 % (w/v) glucose was supplied to 50 mL of LB medium in a 250 mL flask. No exogenous palmitic acid was used because the LCFA transporter had been deleted. After 3 hours, 10 mL supernatant of the culture was obtained by centrifugation, and the concentration of ω-hydroxy palmitic acid was measured by GC.

7. Gas Chromatography Analysis

Sample preparation for product analysis was done as followed: 0.5 mL sample was acidified with 6M HCL to *ca*. pH 2 and extracted with chloroform by vigorous vortexing. They were then converted to their trimethylsilyl (TMS) derivatives by incubating at 50 °C for 20 minutes with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

Qualitative analysis was performed by GC/MS using a TRACE GC

ULTRA gas chromatograph, coupled to an ion trap mass detector ITQ1100. One microliter of the sample was injected by splitless mode (0.8 minute of splitless time) and analyzed using a nonpolar capillary column (5% phenyl methyl siloxane capillary 30 m×250 μm i.d., 0.25 μm film thickness, TR-5ms). Oven temperature started from 50 °C for 1 minute, and increased by 15 °C/min to 250 °C, and held for 10 mins. The temperatures of inlet, mass transfer line, and ion source were 250 °C, 275 °C, 230 °C, respectively. Flow rate of carrier gas(He) was 1.0 mL/min, and the electron energy for the EI mass spectrum was 70 eV. Each peak was identified by comparison of retention time and mass spectrum data of the sample with that of authentic reference.

Quantitative analysis was performed by HP 6890 Series with flame ionization detector(GC/FID). Two microliter of the sample was injected by split mode (split ratio 20.0:1) and analyzed using a nonpolar capillary column (5% phenyl methyl siloxane capillary 30 m×320 μm i.d., 0.25 μm film thickness, HP-5). Oven temperature started from 50 °C for 1 minute, and increased by 15 °C/min to 250 °C, and held for 10 minutes. The temperature of inlet was 250 °C and the temperature of the detector was 280 °C. Flow rate of carrier gas(He)

was 1.0 mL/min, while flow rates of H_2, air, and He in FID were 45.0 mL/min, 400 mL/min, and 20 mL/min, respectively. Each peak was identified by comparison of GC chromatogram with that of authentic reference. Three compounds, palmitic acid, ω-hydroxy palmitic acid, and heptadecanoic acid, were then quantified by making standard curves for each compound (Fig. 3).

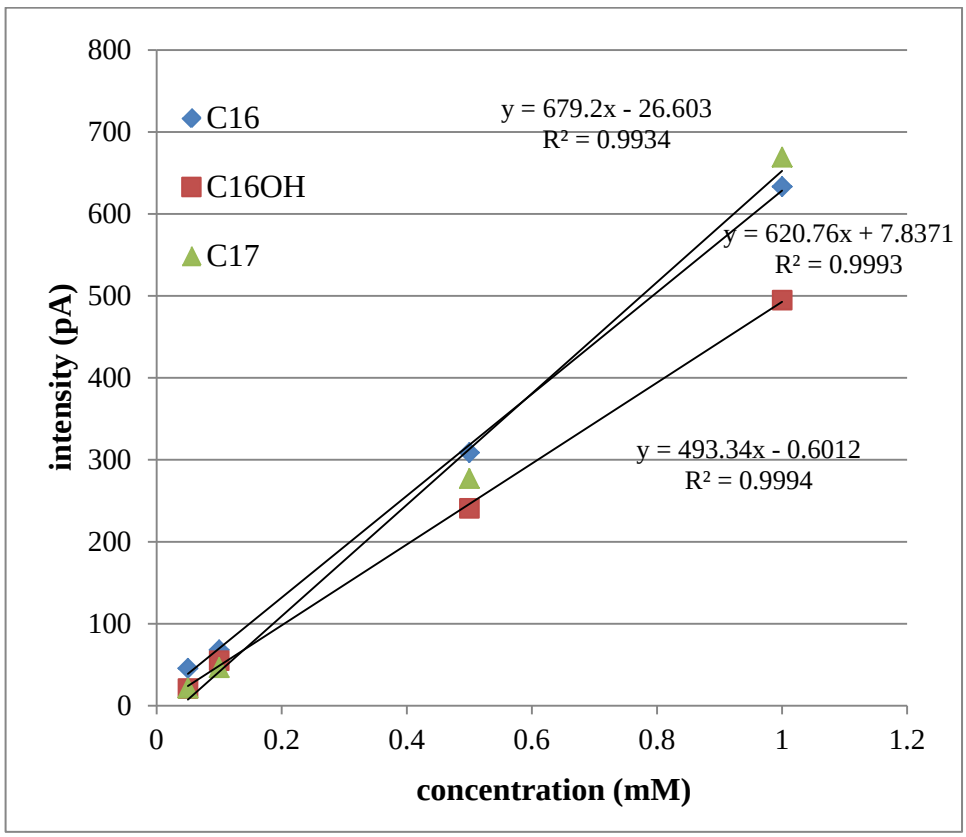

Figure 3. Standard curves for palmitic acid, ω-hydroxy palmitic acid, and heptadecanoic acid.

3. Results

1. *fadD* Deletion Does Not Impair the Ability to Transport LCFA

E. coli FadD not only plays a crucial role in LCFA transport via CoA modification but also initiates degradation of LCFA. Therefore, efficient transport of LCFA without FadD would be desirable to prevent LCFA degradation. From the literature survey (14-16, 30), it was identified that the key role of FadD was maintaining concentration gradient of LCFA across the inner membrane, rather than abstracting LCFA from the inner membrane. This explanation is in good accordance with artificial unilamellar vesicle studies which clarified that LCFA can spontaneously pass through the phospholipid bilayer by adsorption, flip-flop, and desorption (10, 31, 32).

Then, it was hypothesized that if there is a heterologous enzyme which easily consumes LCFA instead of CoA modification by FadD, *fadD* deletion mutant would not have to suffer from the impaired LCFA transport. To confirm this hypothesis, CYP153A, CamA, and

CamB were expressed in the resting cells of *fadD* deletion mutant to convert palmitic acid into ω-hydroxy palmitic acid. Detection of the product indicated successful transport of palmitic acid into the cells. To our surprise, not only hydroxylation activity was found in *fadD* deletion mutant but also the its yield was 31.7 % higher compared to that of the wild-type strain (Fig. 4 (a)).

2. *fadD* Mutant Shows Higher Productivity Despite the Lack of Fatty Acyl-CoA

Since LCFA transport normally takes place in *fadD* deletion mutant, robustness of *fadD* deletion mutant was compared to that of wild-type strain and *fadE* deletion mutant in terms of cell growth and CYP153A expression. The *fadD* deletion mutant might suffer from defected membrane integrity because synthesis of long-chain fatty acyl-CoA is essential for lipid biosynthesis. It is also possible that the *fadD* deletion mutant suffer from impaired LCFA transport through the outer membrane, for long-chain fatty acyl-CoA acts as regulatory signal to upregulate *fad* regulon, which includes LCFA transporter gene *fadL*, by binding to *fad* regulon repressor FadR and inhibiting it

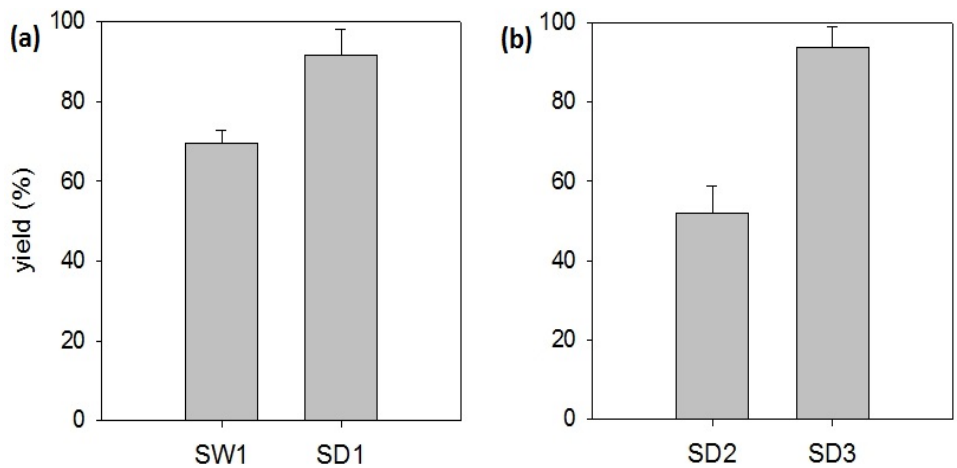

Figure 4. Resting cell reactions to confirm the effect of *fadD* deletion or *fadL* overexpression. (a) SW1 and SD1 were each supplied with 1 mM palmitic acid and cultured for 4 hours. The yield of ω-hydroxy palmitic acid in SD1 was 31.7 % higher than that of SW1. (b) SD2 and SD3 were each supplied with 1 mM palmitic acid and cultured for 2 hours. The yield of ω-hydroxy palmitic acid in SD3 was 80.7 % higher than that of SD2. Every experiment was done in triplicate, and error bars indicate standard deviations.

(33, 34). On the other hand, *fadE* deletion mutant is still able to synthesize long-chain fatty acyl-CoA by *fadD* but has other subsequent side reactions including β-oxidation (Fig. 1).

However, *fadD* deletion mutant showed no disadvantages on dry cell weight (DCW) and CYP153A concentration (Table 2). When glucose was constantly supplied during a fed-batch reaction, *fadD* deletion mutant showed 2-fold higher ω-hydroxy palmitic acid productivity than the wild type strain which is slightly higher than that of *fadE* mutant (Fig. 5).

3. Overexpression of *fadL* Complements *fadD* Deletion Mutant and Expands a Spectrum of Carbon Sources

Because transcription of *fadL* is likely to be repressed in the *fadD* deletion mutant, we cloned *fadL* into a vector for additional expression. When *fadL* was overexpressed in the resting cell of *fadD* deletion mutant, the yield was improved by 80.7 % (Fig. 4 (b)). The hydroxylation reaction was terminated earlier than in the case of *fadD* deletion mutant, because two hours were sufficient enough for *fadD*

Table 2 Dry cell weight and P450 concentration of each strain

	SW1	SD1	SE1
Dry cell weight (g/L) *	13.7	15.3	14.0
P450 concentration (μM) **	0.47	0.53	0.50

* Cells were harvested after 36 hours of fed-batch reaction.
** Volume of the culture was used for concentration calculation.

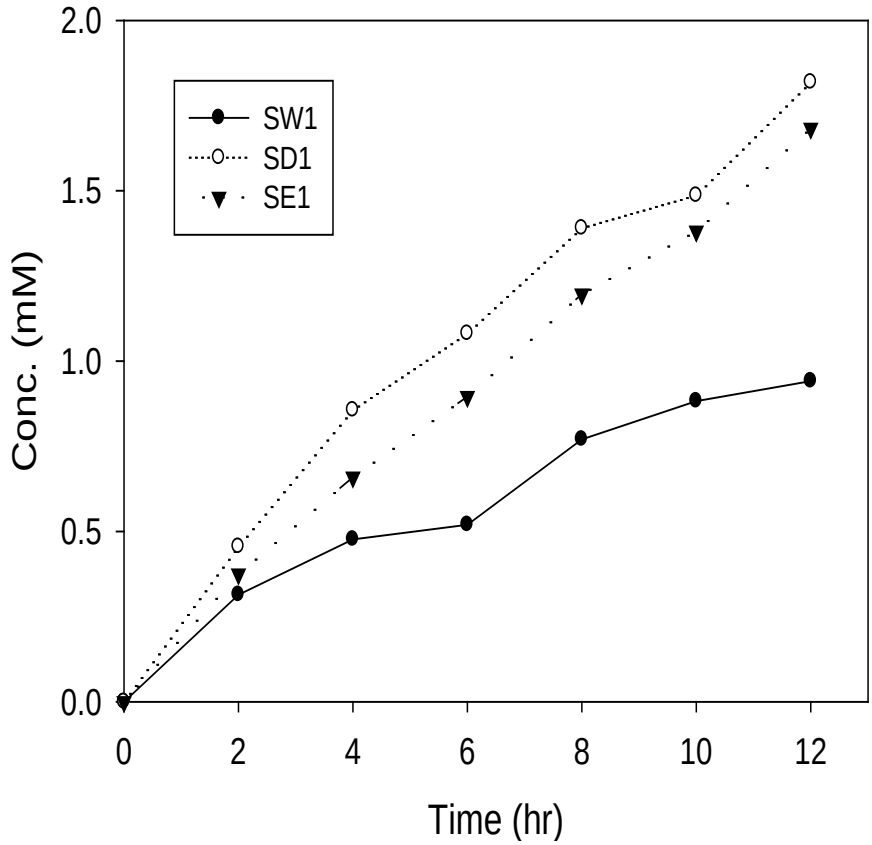

Figure 5. Fed-batch reactions to compare the effects of *fadD* deletion and *fadE* deletion on productivity. SW1, SD1, and SE1 were each supplied with 10 mM palmitic acid and cultured for 12 hours. SD1 showed the highest productivity.

deletion mutant to achieve the maximum yield of ω-hydroxy palmitic acid (data not shown).

The reason for *fadL* overexpression to have the improved yield of ω-hydroxy palmitic acid could be other than LCFA transport by FadL. Recent study on a *Pseudomonas* FadL family protein proved that it is the first bacterial transporter for dihydroxy LCFA secretion (35). Because *Pseudomonas* FadL family protein was found by BLAST search of *E. coli* FadL sequence, further verification on the secretion activity of *E. coli* FadL was required.

CYP153A system was expressed in *fadL* deletion mutant to confirm the detailed role of FadL in ω-hydroxy palmitic acid secretion. Although *fadL* deletion mutant could not transport exogenous palmitic acid into cytoplasm, ω-hydroxy palmitic acid was still synthesized because palmitic acid is the third most abundant LCFA in a natural *E. coli* cell (36). GC analysis indicated that ω-hydroxy palmitic acid was detected in the supernatant of both the wild type and *fadL* deletion mutant (Fig. 6).

Next, effect of *fadL* overexpression on catabolite repression was

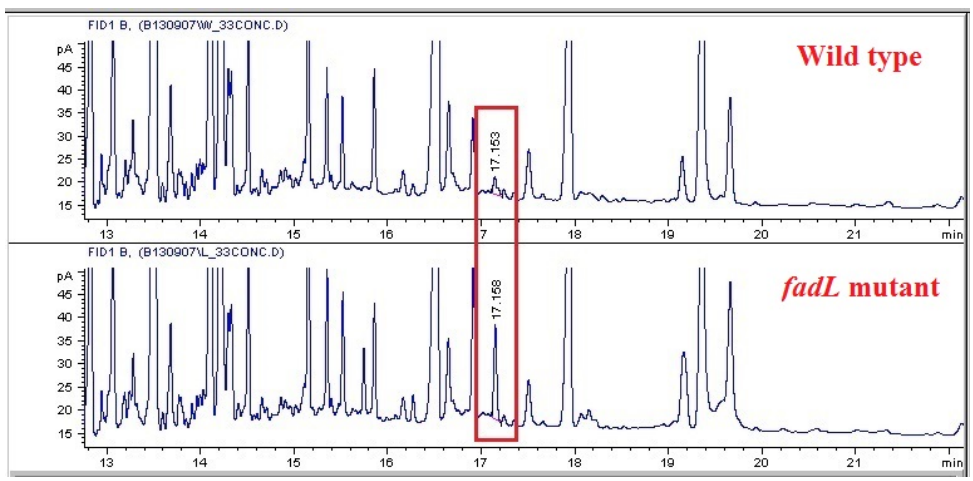

Figure 6. GC chromatogram of ω-hydroxy palmitic acid secretion experiment. Peaks in the block indicate ω-hydroxy palmitic acid. The product was detected in supernatant of cultures of both strains.

tested. Because *fad* regulon for LCFA transport is subject to catabolite repression, the expression levels of Fad enzymes are very low when *E. coli* cells are cultured with rich carbon source like glucose (37). When the effect of *fadL* amplification on LCFA transport was compared between glucose and glycerol, glycerol yielded higher productivity than glucose in negative control, whereas glucose yielded higher productivity when *fadL* was overexpressed, reflecting catabolite repression (Fig. 7).

4. Combination of *fadD* Deletion and FadL Overexpression Increased the Productivity

Finally, the effect of *fadD* deletion and *fadL* overexpression on the production of ω-hydroxy palmitic acid was combined to see how much the productivity was improved (Fig. 8). A new plasmid harboring both *CYP153A* and *fadL* was constructed, and the transformed strain was cultured in a shake flask for fed-batch reaction. After 36 hours of the reaction with glucose and pH control as indicated in materials and methods section, 2.4 g/L of ω-hydroxy palmitic acid was obtained, resulting 5.5-fold higher than that of wild-

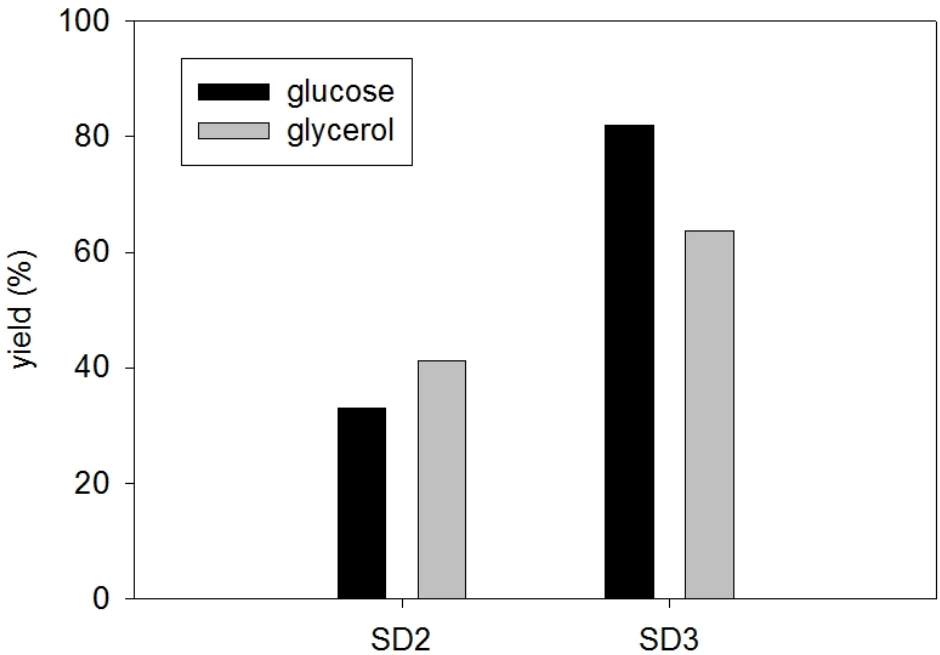

Figure 7. Resting cell reaction to confirm the effect of FadL overexpression on catabolite repression. SD2 and SD3 were cultured with either 1 % glucose or glycerol for 6 hours before harvest. Glucose was used to induce catabolite repression before converting 1 mM palmitic acid. Glucose media resulted in lower yield when fadL was only expressed from *E. coli* chromosome, but the overexpression of *fadL* from an additional vector reversed the result.

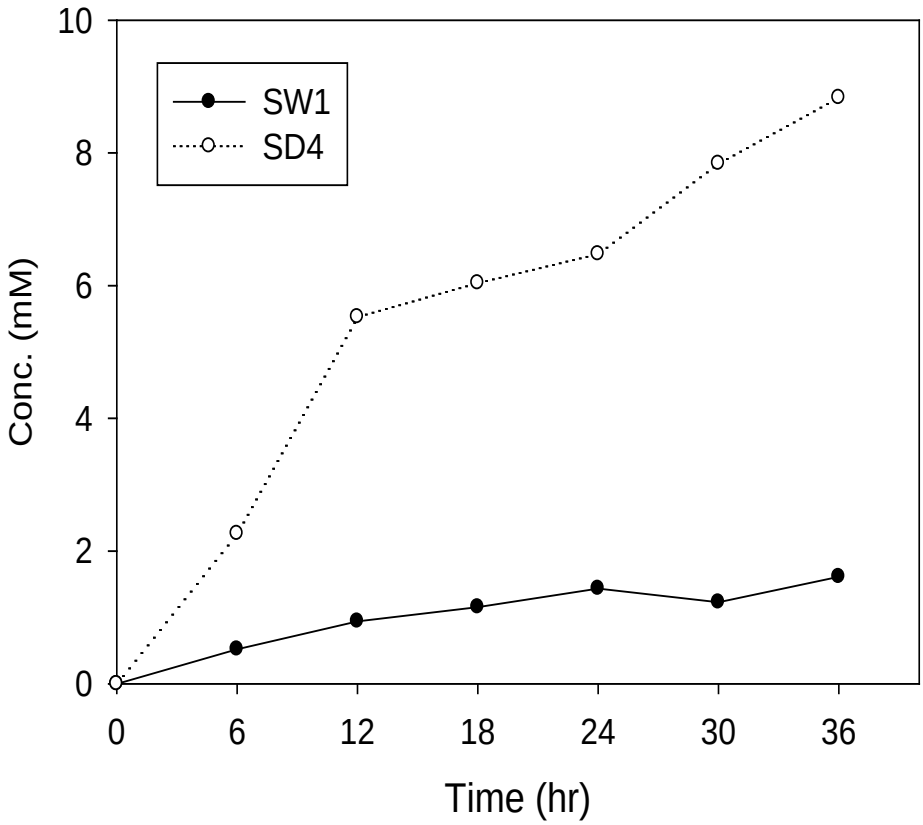

Figure 8. Fed-batch reaction to confirm the effect of *fadD* deletion and FadL overexpression combination. SW1 and SD4 were each cultured with 15 mM palmitic acid. SD4 showed 5.5-fold higher productivity than SW1.

type strain.

4. Discussion

To maximize ω-hydroxy palmitic acid productivity by preventing palmitic acid and ω-hydroxy palmitic acid degradation, the possibility of LCFA transport without FadD was examined. When CYP153A was expressed in *fadD* deletion mutant, the hydroxylation activity occurred which implied LCFA transport (fig 4 (a)). This result proved that the hypothesis that a heterologous enzyme which consumes LCFA can replace FadD in LCFA transport was correct. Because LCFA is accumulated in the periplasm by natural FadL and consumed in the cytoplasm by CYP153A, concentration gradient which drives diffusion must have been maintained across the inner membrane. This result of *fadD* deletion mutant, however, does not deny the existing LCFA transport mechanism that LCFA is abstracted from the inner membrane by FadD. Membrane structure could have been altered because lipid composition is different in *fadD* deletion mutant (38). This is not surprising since LCFA-CoA synthesized by FadD is known to be used in lipid biosynthesis. There is not enough evidence yet to determine whether FadD actually abstracts LCFA from the inner

membrane or not.

The lack of LCFA-CoA in *fadD* deletion mutant could be a double-edged sword for fermentation since growth defect of *fadD* deletion mutant had been already reported (28). It was not easy to predict which effect would be predominant: negative effect coming from the growth defect, disturbed membrane integrity, and *fadL* repression, or positive effect resulting from prevention of side pathways. Productivity and DCW of *fadD* deletion mutant were thus compared with those of wild-type strain and *fadE* deletion mutant which can biosynthesis LCFA-CoA for the lipid biosynthesis but does not have β-oxidation pathway. Interestingly, DCW of *fadD* deletion mutant was higher than that of wild-type strain and *fadE* deletion mutant when abundant palmitic acid (10 mM) was present (table 2). Two aspects of *fadD* deletion mutant can be inferred from the result; first, the amount of LCFA synthesized by elongation within the cell is enough for lipid biosynthesis of growing cell. Second, ATP spared from excessive CoA ligation can be used for extra cell growth. Also, the fact that *fadD* deletion mutant showed the highest ω-hydroxy palmitic acid productivity (Fig. 5) implied even the penalties

mentioned above were not enough to predominate over the LCFA side pathway issue. This experiment clearly demonstrated that *fadD* deletion mutant has prospect to be the host strain for LCFA hydroxylation.

Although *fadD* deletion mutant showed the highest productivity, there was still room for improvement. To offset the inability to inhibit FadR repressor, which represses transcription of *fadL*, in *fadD* deletion mutant, *fadL* was then overexpressed from an additional vector. When *fadL* was amplified in the background of *fadD* deletion mutant with CYP153A P450 system, productivity of ω-hydroxy palmitic acid was increased as we expected (Fig. 4 (b)). This suggested the increased uptake of palmitic acid resulted in additional hydroxylation reaction. This notion required further verification because it was possible that the improvement is attributed to better secretion of ω-hydroxy palmitic acid by FadL (35). However, *fadL* deletion mutant was also able to secrete ω-hydroxy palmitic acid (Fig. 6), indicating that FadL does not participate in the secretion of ω-hydroxy palmitic acid. Therefore, it was concluded that the productivity improvement of ω-hydroxy palmitic acid resulting from

fadL overexpression solely came from increased palmitic acid transport.

Overexpression of *fadL* had another advantage in choosing carbon sources. When there was no *fadL* overexpression in *fadD* deletion mutant, glycerol media yielded more ω-hydroxy palmitic acid than glucose media although glucose is the better carbon source (Fig. 7). This might have been caused by catabolite repression induced by glucose which is proved to repress *fad* regulon (39). However, overexpression of *fadL* reversed the result and glucose media yielded higher productivity of ω-hydroxy palmitic acid. This suggested that *fadL* overexpression from the additional vector dominate the effect of catabolite repression because *fadL* in the vector is not regulated by catabolite repression. The *fadL* overexpression strategy allowed a better choice of carbon source by separating the level of FadL from catabolite repression.

5. Conclusion

The dilemma of LCFA hydroxylation using *E. coli* was that transport and degradation of LCFA is closely linked by FadD. Here, we showed a method to maintain the ability to transport LCFA when *fadD* which encodes FadD is deleted. Although *fadD* deletion mutant was a robust host strain for LCFA hydroxylation with high DCW and productivity, the disadvantage of using *fadD* deletion mutant appeared to be the repression on *fadL* by FadR. To overcome the repression, *fadL* was overexpressed from an additional vector. This strategy also allowed us to overcome catabolite repression, making it easier to choose adequate carbon source. The combination of the deletion of *fadD* and the overexpression of *fadL* resulted to yield 5.5 times higher ω-hydroxy palmitic acid productivity than wild-type strain. The genetic manipulation of the two endogenous genes is simple enough to be done in any laboratory and can be used for other substrates and enzymes because FadD and FadL act on not only palmitic acid but also other LCFAs.

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국문 초록

긴사슬지방산은 친수성의 카복실기와 소수성의 탄소 사슬을 지니기 때문에 다양한 물질의 전구체이다. 긴사슬지방산에 하이드록시기를 붙이는 효소들은 대장균에서 발현되어 유용한 물질을 만드는 데에 사용되었다. 긴사슬지방산을 이러한 물질로 바꾸는 데 있어서 가장 큰 문제는 긴사슬지방산의 수송과 분해가 FadD에 의해 하나로 묶여있다는 점이다. 우리는 베타 산화를 포함하여 긴사슬지방산의 분해를 막으면서도 수송에는 영향을 주지 않는 효과적인 방법을 개발하였다. 이것은 두 개의 고유단백질인 FadD와 FadL의 발현을 조절함으로써 이루어졌다. 우선, 긴사슬지방산을 소모하는 외래효소가 긴사슬지방산 수송 과정에서 FadD의 역할을 대체할 수 있다는 것을 밝혔다. *Marinobacter aquaeolei* 유래의 CYP153A와 CamA, CamB를 이용한 반응이 모델 시스템으로 사용되었다. *fadD* 돌연변이는 긴사슬지방산과 그 유도체의 안정성은 보장했지만, FadR 억제 작용을 저해하지 못 하기 때문에 추가적인 보완을 필요로 하였다. FadL의 과발현은 FadR 억제 작용뿐만 아니라 이화물질억제도 극복하게 하여서 탄소 공급원 선택의 폭을 넓혔다. *fadD* 돌연변이에서 FadL를 과발현 시켰을 때, 최종적으로 오메가-하이드록시 팔미트산은 2.4 g/L가 생산되었다. 이것은 야생형 균주보다 5.5배 향상된 결과이다. 이 방법은 대장균을 이용한 모든 긴사슬지방산의 수산화 반응에 적용될 수 있다. 또한, 두 개의 대장균 고유유전자만을 조절하기 때문에 어떤 연구실에서도 손

쉽게 사용할 수 있다.

주요어: 수산화 긴사슬지방산, 긴사슬지방산 수산화, 긴사슬지방산 분해, 긴사슬지방산 수송, FadD, FadL