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Intracellular transformation rates of fatty acids are influenced by expression of the fatty acid transporter FadL in *Escherichia coli* cell membrane

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ABSTRACT

Fatty acids have a low permeability through the cell membrane. Therefore, the intracellular biotransformation of fatty acids can be slow due to supply limitations. The effects of expression level of the fatty acid transporter FadL in *Escherichia coli* on the biotransformations were investigated. The enhanced expression of FadL led to 5.5-fold increase of the maximum reaction rate V_{max} (i.e., 200 μmol/min per g dry cells (200 U/g dry cells)) of the recombinant *E. coli* expressing a hydratase of *Stenotrophomonas maltophilia* in the periplasm with respect to hydration of oleic acid. The FadL expression level was also critical for oxidation of 12- and 10- hydroxyoctadecanoic acid by the recombinant *E. coli* expressing an alcohol dehydrogenase (ADH) of *Micrococcus luteus*. In addition, the multistep biotransformation of ricinoleic acid into the ester (i.e., (*Z*)-11-(heptanoyloxy) undec-9-enoic acid) by the recombinant *E. coli* expressing the ADH of *M. luteus* and a Baeyer-Villiger monooxygenase of *Pseudomonas putida* KT2440 was 2-fold increased to 40 U/g dry cells with expression of FadL to an appropriate level. The FadL expression level is one of the critical factors to determine whole-cell biotransformation rates of not only long chain fatty acids but also hydroxy fatty acids. This study may contribute to whole-cell biocatalyst engineering for biotransformation of hydrophobic substances.

1. Introduction

One of the hurdles in whole-cell biotransformation of hydrophobic molecules may include transport through the cell membrane to reach the intracellular enzymes. This is mainly because of poor solubility and dispersibility of the reaction substrates in the aqueous environment and of low permeability of the cellular membranes (Jeon et al., 2016; Nikaido, 2003; Schrewe et al., 2013; Willrodt et al., 2015). For instance, the outer layer of outer membrane of *Escherichia coli*, which consists mostly of lipopolysaccharides, was reported as a major barrier to transport of hydrophobic compounds into *E. coli* (Nikaido, 2003).

There could be a number of ways to increase mass transport efficiency of hydrophobic substances for the whole-cell biotransformations. One approach was to use surfactants to allow dispersion of the hydrophobic substrates into the reaction medium (Pfruender et al., 2006). Another way was to increase cell membrane permeability to facilitate transport of the reaction substrates into the cells (Chen, 2007; Delcour, 2009; Lee et al., 2011; Ni and Chen, 2004). The membrane permeability could be enhanced by treatment of the whole-cells with organic chemicals and solvents which may reduce integrity of cellular membranes (Leon et al., 1998). However, these methods may negatively affect cell viability and metabolic activity, which is critical for cofactor-dependent whole-cell biotransformations (Haberland et al., 2002; Julsing et al., 2012a; Lee et al., 2015; Trudgill, 1990). The cell surface display of target enzymes (Jose et al., 2002; Schumacher et al., 2012; Yim et al., 2010) is also not so appropriate for the cofactor-dependent biocatalysis because of difficulty in cofactor regeneration.

Recently, an elegant method to improve mass transport efficiency of the hydrophobic substrates to *E. coli* cells was reported. It was to use a transporter protein of the hydrophobic molecules through the cell membrane. For instance, the AlkL from *Pseudomonas putida* GPo1, which is involved in transport of alkanes into bacterial cells, was demonstrated to facilitate transport of not only aliphatic alkanes (i.e. C7-C16 alkanes) but also fatty acid methyl esters (i.e. nonanoic acid methyl

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ester, dodecanoic acid methyl ester) and monoterpenes (e.g., (*S*)-limonene) into *E. coli* cells (Cornelissen et al., 2013; Grant et al., 2014; Julsing et al., 2012b; Scheps et al., 2013; Schrewe et al., 2014). However, the AlkL was not active with fatty acids.

The fatty acids in particular long chain fatty acids (LCFAs) are transported into *E. coli* via a two-step process. Firstly, LCFAs cross the outer membrane through the long-chain fatty acid transporter FadL (DiRusso and Black, 1999; Maloy et al., 1981; Van den Berg et al., 2004). Secondly, the LCFAs enter the plasma membrane and are activated by a membrane-associated fatty acyl-CoA synthetase and released into the cytosol (DiRusso and Black, 1999; Maloy et al., 1981). Crossing the plasma membrane was assumed to be driven via flip-flop of the fatty acid molecules in bilayer membranes (Wei and Pohorille, 2014).

This study has focused on effects of expression level of FadL in the cell membranes of *E. coli* on whole-cell biotransformation of fatty acids. Biotransformation of not only the representative substrates of FadL (e.g., oleic acid), but also the hydroxy fatty acids (e.g., 10-hydroxyoctadecanoic acid, 12-hydroxyoctadecanoic acid, ricinoleic acid (12-hydroxyoctadec-9-enoic acid), and 5-hydroxydecanoic acid) was examined. We have also investigated effect of FadL expression level on multi-step whole-cell biotransformation of ricinoleic acid into the ester (i.e., (*Z*)-11-(heptanoyloxy)undec-9-enoic acid) via 12-keto-octadec-9-enoic acid (Song et al., 2013) (Scheme S1).

2. Materials and methods

2.1. Microbial strains and culture medium

Recombinant *E. coli* cells were cultivated in lysogeny broth (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) supplemented with appropriate antibiotics for plasmid DNA preparation and seed cultivation (Table S1). The Riesenberg medium (Riesenberg, 1991) supplemented with 10 g/L glucose and the appropriate antibiotics was used for the main cultivation and whole-cell bioconversion. The Riesenberg medium contained 4 g/L $(NH_4)_2HPO_4$, 13.5 g/L KH_2PO_4, 1.7 g/L citric acid, 1.4 g/L $MgSO_4$, and 10 ml/L trace metal solution (10 g/L $FeSO_4$, 2.25 g/L $ZnSO_4$, 1.0 g/L $CuSO_4$, 0.5 g/L $MnSO_4$, 0.23 g/L $Na_2B_4O_7$, 2.0 g/L $CaCl_2$, and 0.1 g/L $(NH_4)_6Mo_7O_{24}$). Recombinant gene expression was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and/or 2 g/L rhamnose to the culture broth at the exponential growth phase (optical density at 600 nm OD_{600} : 0.5–0.6) followed by a 20 h incubation at 20 °C.

2.2. Chemicals and materials

Ricinoleic acid, rhamnose, and N-methyl-N-(trimethylsilyl) trifluoroacetamide were purchased from Tokyo Chemical Co (Tokyo, Japan). Glucose was purchased from Junsei Chemical Co (Tokyo, Japan). Oleic acid, palmitic acid, n-heptanoic acid, pyridine, hexane, antibiotics, trace elements for culture medium, IPTG, and Tween80 were purchased from Sigma (St. Louis, MO, USA). Ethyl acetate was purchased from Duksan Pure Chemical Co. (Ansan, Republic of Korea). FadL antibody was obtained from Abmart (Shanghai, China). 10-Hydroxyoctadecanoic acid was prepared in our lab according to the previous study (Song et al., 2013).

2.3. Plasmid construction and genome engineering

The *ohyA* gene including the *pelB* signal sequence (*pelBSS*) was amplified by polymerase chain reaction (PCR) using pET26b-OhyA as previous reported (Jung et al., 2015). The *pelBSS-ohyA* gene fragment was inserted into pACYCduet vector (Invitrogen) resulting in pACYC-PelBSS-OhyA by sequence ligation independent cloning (SLIC) method (Li and Elledge, 2012).

Genomic DNA was extracted from *E. coli* BL21(DE3) grown in LB medium using the Qiagen Genomic DNA Purification kit (Qiagen, Valencia, CA, USA). The full-length *fadL* gene (GenBank accession code: CAQ32746.1) was PCR-amplified from the genomic DNA and subcloned into pETduet vector of the Nde1 site resulting in pET-FadL (Table S1). The *fadL* gene was amplified by PCR using pET-FadL as the template with Duet_fadL_EcoRV_F and Duet_fadL_EcoRV_R as primers (Table S2). They were inserted into the EcoRV site of the pACYC-ADH (Song et al., 2013) resulting in the vector pACYC-ADH-FadL. For construction of pJOE-BVMO-FadL, the *fadL* gene was amplified by PCR using pET-FadL as the template with pJOE-fadL_NdeI_F and pJOE-fadL_BamHI_R as the primers. They were inserted into the NdeI and BamHI site of the pJOE vector (Rehdorf et al., 2007) resulting in the vector pJOE-FadL. The DNA fragment including the rhamnose promoter region to *fadL* gene was amplified with pJOE-BVMO_fadL_BamHI and pJOEBVMO_fadL_HindIII as the primers using pJOE-FadL as the template. They were inserted into BamHI and HindIII site of pJOE-BVMO (Rehdorf et al., 2007). The PCR-amplified DNA sequence was verified by sequencing (Macrogen, Seoul, Korea). The *fadL* gene knock-out mutant was constructed by the method described by Datsenko and Wanner (Datsenko and Wanner, 2000). The plasmids, strains and primers used in this study are presented in Table S1 and S2.

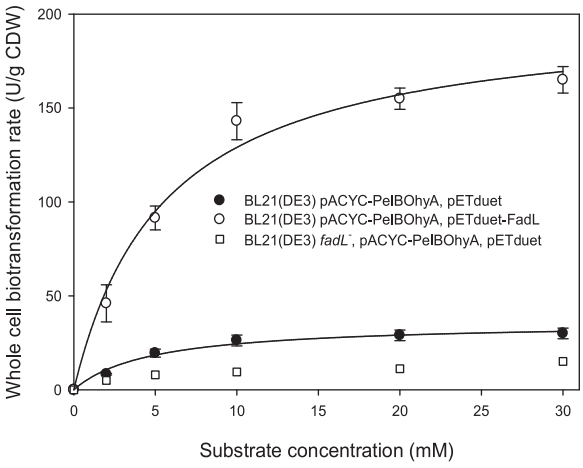

Fig. 1. The oleic acid hydration kinetics of recombinant *E. coli* BL21(DE3) Δ*fadL* pACYC-PelBSS-OhyA, pET-duet, *E. coli* BL21(DE3) pACYC-PelBSS-OhyA, pET-duet, and *E. coli* BL21(DE3) pACYC-PelBSS-OhyA, pET-FadL, expressing a fatty acid double bond hydratase gene (i.e., *ohyA*) of *Stenotrophomonas maltophilia* in the periplasm. The whole cell biotransformations were initiated at the stationary phase (cell density: 3 g CDW/L) by adding oleic acid and Tween80 into the culture broth at 35 °C. The experiments were performed in triplicate. Error bars indicate standard deviations.

Table 1
Kinetic constants of recombinant *E. coli*-based biocatalysts.[a]

Whole-cell Biocatalysts	K_S (mM)	V_m (U/g CDW)
E. coli BL21(DE3) pACYC-PelBSS-OhyA, pET-	4.7 ± 0.9	36 ± 2.2
E. coli BL21(DE3) pACYC-PelBSS-OhyA, pET-FadL	5.6 ± 0.9	200 ± 9.9

[a] The initial whole-cell hydration rates of oleic acid were determined by measuring the 10-hydroxyoctadecanoic acid concentrations with GC/MS over reaction times at 35 °C. The experiments were carried out in triplicate.

2.4. Whole cell biotransformation

Whole cell biotransformations were carried out according to our previous studies (Cha et al., 2018; Jeon et al., 2012; Song et al., 2016; Sudheer et al., 2017). Briefly, the recombinant *E. coli* cells were cultivated in Riesenberg medium at 37 °C and expressions of target genes were induced with 0.1 mM IPTG and/or 2.0 g/L rhamnose depending on the promoter (Table S1). Afterwards, cultivation temperature was reduced to 20 °C to facilitate soluble expression of the target genes.