Identification and Characterization of a New Enoyl Coenzyme A Hydratase Involved in Biosynthesis of Medium-Chain-Length Polyhydroxyalkanoates in Recombinant *Escherichia coli*

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The biosynthetic pathway of medium-chain-length (MCL) polyhydroxyalkanoates (PHAs) from fatty acids has been established in *fadB* mutant *Escherichia coli* strain by expressing the MCL-PHA synthase gene. However, the enzymes that are responsible for the generation of (R)-3-hydroxyacyl coenzyme A (R3HA-CoA), the substrates for PHA synthase, have not been thoroughly elucidated. *Escherichia coli* MaoC, which is homologous to *Pseudomonas aeruginosa* (R)-specific enoyl-CoA hydratase (PhaJ1), was identified and found to be important for PHA biosynthesis in a *fadB* mutant *E. coli* strain. When the MCL-PHA synthase gene was introduced, the *fadB maoC* double-mutant *E. coli* WB108, which is a derivative of *E. coli* W3110, accumulated 43% less amount of MCL-PHA from fatty acid compared with the *fadB* mutant *E. coli* WB101. The PHA biosynthetic capacity could be restored by plasmid-based expression of the *maoC* gene in *E. coli* WB108. Also, *E. coli* W3110 possessing fully functional β-oxidation pathway could produce MCL-PHA from fatty acid by the coexpression of the *maoC* gene and the MCL-PHA synthase gene. For the enzymatic analysis, MaoC fused with His\(_6\)-Tag at its C-terminal was expressed in *E. coli* and purified. Enzymatic analysis of tagged MaoC showed that MaoC has enoyl-CoA hydratase activity toward crotonyl-CoA. These results suggest that MaoC is a new enoyl-CoA hydratase involved in supplying (R)-3-hydroxyacyl-CoA from the β-oxidation pathway to PHA biosynthetic pathway in the *fadB* mutant *E. coli* strain.

Polyhydroxyalkanoates (PHAs) are polyesters of (R)-hydroxyalkanoic acids accumulated in numerous bacteria as an energy and carbon storage material under nutrient limiting condition in the presence of excess carbon source (1, 17, 20). PHAs have been attracting much attention as they can be used as biodegradable polymers (20) and as the sources of chiral monomers (18). For example, in short-chain-length-PHA-producing bacteria such as *Ralstonia eutropha* and *Alcaligenes latus*, two acetyl coenzyme A (acetyl-CoA) moieties from various carbon sources are condensed to acetoacetyl-CoA by 3-ketothiolase (PhaA) and sequentially converted to (R)-3-hydroxybutyryl-CoA (R3HB-CoA) by acetoacetyl-CoA reductase (PhaB). Then, R3HB-CoA is added to the growing chain of poly(3-hydroxybutyrate) [P(3HB)] by the short-chain-length PHA synthase (PhaC) (29).

In pseudomonads belonging to the rRNA homology group I, the intermediates of fatty acid metabolism including enoyl-CoA, (S)-3-hydroxyacyl-CoA, 3-ketoacyl-CoA, and 3-hydroxyacyl-acyl carrier protein (ACP) are major precursors for medium-chain-length (MCL) PHAs (17, 20, 36). The metabolic links between the fatty acid metabolism and PHA biosynthesis are mediated by various enzymes such as enoyl-CoA hydratase (7, 8, 9, 23, 34, 35), 3-ketoacyl-ACP reductase (22, 27, 33), epimerase (20), and 3-hydroxyacyl-ACP:CoA transacylase (12, 26). The genes encoding these enzymes have been cloned from various bacteria and characterized in detail at the molecular level. Recently, the MCL-PHA biosynthesis pathway was successfully established in recombinant *Escherichia coli* by expressing the MCL-PHA synthase gene. The β-oxidation pathway has been engineered by the overexpression of enoyl-CoA hydratase (34, 35) or 3-ketoacyl-ACP reductase (22, 27, 33), and/or by the disruption of FadB or FadA (15, 22, 24, 25, 27). In the former case, the metabolic connection of β-oxidation pathway to PHA biosynthesis is quite clear. However, in the latter case there must exist unidentified enzymes in *E. coli* which convert β-oxidation intermediates to (R)-3-hydroxyacyl-CoA (R3HA-CoA) when the function of FadB or FadA is disrupted. There has been a report showing that the overexpression of *Pseudomonas oleovorans* fadBA genes could not establish the PHA biosynthetic pathway in recombinant *E. coli* (7). Recently, YfcX, which is homologous to FadB, was found to be necessary for the MCL-PHA formation in a *fadB* mutant *E. coli* strain (30).

These results encouraged us to search for other missing enzymes linking the β-oxidation pathway and the PHA biosynthetic pathway in the *fadB* mutant *E. coli*. Through the *E. coli* protein sequence database search, MaoC, which is homologous to the *P. aeruginosa* (R)-specific enoyl-CoA hydratase (PhaJ1) was identified. Results obtained by the inactivation of the chro-
molecular analysis of MaOC suggested that MaOC is a newly identified enoyl-CoA hydratase, which is involved in linking the β-oxidation and the PHA biosynthetic pathway in the fadB mutant E. coli strain.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. E. coli XL1-Blue (Stratagene Cloning Systems, La Jolla, Calif.) and DH5α (Invitrogen Life Technologies, Carlsbad, Calif.) were used as host strains for general cloning and the synthesis of MCL-PHAs. The fadB (WB101 maoC::Tc), and WB112 (WB101 yfcX::Tc) strains were constructed by replacing the kanamycin resistant gene obtained from pACYC177 (New England Biolabs, Beverly, Mass.) into the middle of fadB gene obtained from pBBR1MCS (14).

Plasmid construction. PCR was performed with the PCR Thermal Cycler MP (Takara Shuzo Co., LTD., Shiga, Japan) using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). DNA sequencing was carried out using the BigDye Terminator cycle sequencing kit (Perkin-Elmer Co., Boston, Mass.). Taq polymerase, and ABI Prism 377 DNA sequencer (Perkin-Elmer Co.). All DNA manipulations including restriction digestion, ligation, and agarose gel electrophoresis were carried out by standard procedures (28).

Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
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<tr>
<td>E. coli strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>recA1 endA1 gxtA69 thi hsdR17 supE44 relA1 λ- lac F' [proAB lacI5 lacZΔM15 Tn10 (Tet')]</td>
<td>Stratagene*</td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (Δ880lacZΔM15) hsdR17 recA1 endA1 gxtA69 thi- relA1</td>
<td>Stratagene*</td>
</tr>
<tr>
<td>W3110</td>
<td>F- mcrA mcrB IN(rrnD mrr) E1 λ-</td>
<td>LTI†</td>
</tr>
<tr>
<td>WB101</td>
<td>W3110 (fadB::Km)</td>
<td>This study</td>
</tr>
<tr>
<td>WB108</td>
<td>W3110 (fadB::Km maoC::Tc)</td>
<td>This study</td>
</tr>
<tr>
<td>WB112</td>
<td>W3110 (fadB::Km yfcX::Tc)</td>
<td>This study</td>
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<tr>
<td>Plasmids</td>
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<td>Cm'; cloning vehicle</td>
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<tr>
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<td>pT799A derivative; gntT104 promoter; Ap'</td>
<td>22</td>
</tr>
<tr>
<td>pTac99A</td>
<td>pT799A derivative; lac promoter; Ap'</td>
<td>This study</td>
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<tr>
<td>pMCS104613C2</td>
<td>pBR1MCS derivative; gntT104 promoter, phaC2p</td>
<td>This study</td>
</tr>
<tr>
<td>p10499MaoC</td>
<td>p10499A derivative; maocCp</td>
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</tr>
<tr>
<td>pTac99MaoCII</td>
<td>pTac99A derivative; maocCp-His6 tag</td>
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<tr>
<td>p10499B2341</td>
<td>p10499A derivative; yfcXEc</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Stratagene Cloning System.
† Invitrogen Life Technologies.
‡ Korean Collection for Type Cultures, Daejeon, Republic of Korea

Table 2. Primers used in PCR experiments

<table>
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<tr>
<th>Primer no.</th>
<th>Sequence</th>
<th>Gene</th>
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<tr>
<td>1</td>
<td>5'-TTTCCGAGCTCATGACGAGTTAGCCAGTTC</td>
<td>maocCp</td>
</tr>
<tr>
<td>2</td>
<td>5'GCTCTGAGTAAATTCGACAATACCGTG</td>
<td>maocCp</td>
</tr>
<tr>
<td>3</td>
<td>5'-TTTCCGAGCTCATGACGAGTTAGCCAGTTC</td>
<td>maocCp-His6 tag</td>
</tr>
<tr>
<td>4</td>
<td>5'-GCTCTGAGTAAATTCGACAATACCGTG</td>
<td>maocCp-His6 tag</td>
</tr>
<tr>
<td>5</td>
<td>5'-GGAATTCATGAAATACGATCCGTTTTACC</td>
<td>yfcXEc</td>
</tr>
<tr>
<td>6</td>
<td>5'-CCCAGGTTTTATCAGCAAGTGTTG</td>
<td>yfcXEc</td>
</tr>
</tbody>
</table>

* Restriction enzyme sites are shown in boldface type. The template for all primers was the E. coli W3110 chromosome.

Plasmids and primers used in this study are listed in Table 1 and 2, respectively. Plasmid pMCS104613C2 was constructed by the insertion of EcoRV-SspI digested gene fragment of p10499613C2 (22) containing the gntT104 promoter and the Pantromonas sp. strain 61-3 phaC2p gene (21) into EcoRV-digested pBBR1MCS (14).

Primers for the amplification of the maocCp and yfcXEc genes were designed based on the reported E. coli genome sequence (3). A plasmid for the expression of the E. coli maocCp gene was constructed by the insertion of the PCR amplified maocCp gene at SacI and XbaI sites of plasmid p10499A (22). Also, PCR amplified yfcXEc gene was inserted into p10499A at EcoRI and HindIII sites. pTac99A is a derivative of pT799A (Pharmacia Biotech., Uppsala, Sweden), which was constructed by replacing the lac promoter of pT799A with the tac promoter from pKK223-3 (Pharmacia Biotech.) digested by PstI and EcoRI. pTac99MaoCH was constructed by the insertion of PCR amplified maocCp gene fused with His6-Tag at its C-terminal (maocCp-His6 tag) into pTac99A at the SacI and XbaI sites.

Culture conditions. E. coli XL1-Blue and DH5α were cultured at 37°C in Luria-Bertani (LB) medium (containing, per liter, 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl). For the biosynthesis of PHA, recombinant E. coli W3110, WB101, WB108, and WB112 strains were cultivated for 96 h in 250-ml flasks containing 100 ml of LB medium supplemented with sodium decanoate (2 gliter; Sigma Co., St. Louis, Mo.). Flask cultures were carried out in a rotary shaker at 250 rpm and 30°C. Ampicillin (50 mg/liter) and chloramphenicol (34 mg/liter) were added to the medium.

Analytical procedures. PHA concentration and monomer composition were determined by gas chromatography (Donam Co., Seoul, Korea) equipped with a fused silica capillary column (SPB-5 film [30 m by 0.32 mm; inner diameter, 0.25 μm]; Supelco, Bellefonte, Pa.) using benzoic acid as an internal standard (4). Cell concentration, defined as dry cell weight (DCW) per liter of culture broth, was determined as previously described (6, 15). The residual cell concentration was defined as the cell concentration minus PHA concentration. The PHA content...
Identification of the E. coli gene homologous to P. aeruginosa phaJ1<sub>Pa</sub>. The E. coli mao<sub>Ec</sub> gene was found to be homologous to the P. aeruginosa phaJ1<sub>Pa</sub> gene by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). From the conserved domain database, PhaJ1 was found to have the MaoC like domain. The gene was found to be homologous (weight percent) to the E. coli mao<sub>Ec</sub> gene by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). From the conserved domain database, PhaJ1 was found to have the MaoC like domain. The catalytically important residues Asp and His are indicated by arrows.
3HB monomer (21). The mole fraction of 3HO was the highest when recombinant E. coli phaC2Ps expression (31, 32). Until now, the enzymatic characterization of 3-hydroxybutyrate (3HB) was not incorporated into PHA when the MCL-PHA synthase (15, 22, 24, 25, 27). The elucidated metabolic pathways for the production of MCL-PHA from the intermediates of β-oxidation pathway are shown in Table 3. Various enzymes including FadD, FadE, enoyl-CoA hydratase, epimerase and 3-ketoacyl-CoA reductase are involved in the generation of R3HA-CoAs, the substrates for PHA synthase, from fatty acid (Fig. 3).

It has been shown that the generation of R3HA-CoAs is possible only when the multienzyme complex FadAB is partially or fully inactivated. The enzymes responsible for connecting the β-oxidation and the PHA biosynthetic pathways in E. coli have not been thoroughly elucidated yet. Only recently, YfcX, which is homologous to FadB, has been suggested to be responsible for supplying MCL-PHA precursors from the β-oxidation pathway when FadB activity is removed in recombinant E. coli (30). We, therefore examined whether the overexpression of the yfcXEc gene can establish PHA biosynthetic pathway in E. coli W3110 harboring the Pseudomonas sp. strain 61-3 MCL-PHA synthase gene. However, PHA was not accumulated from sodium decanoate (Table 3). Also, there has been a report showing that the overexpression of the P. oleovorans fadBA genes did not support PHA biosynthesis in recombinant E. coli (7). The effect of coexpressing the yfcXEc gene was notable in a fadB mutant E. coli WB101, resulting in the increase of PHA concentration compared with that obtained in E. coli WB101 harboring the phaC2Ps gene only (Table 3).

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Protein database search revealed that a putative aldehyde dehydrogenase, MaoC, is homologous to the P. aeruginosa enoyl-CoA hydratase (PhaJ1). The maoC gene exists as an operon with the maoA gene in E. coli (31, 32). The mao operon has been reported to encode enzymes involved in the degradation of aromatic amine compounds. The maoA gene encodes an aromatic amine oxidase, which is similar to that of Klebsiella aerogenes (31, 32). Until now, the enzymatic characterization of E. coli MaoC has not been carried out. We, therefore, examined whether this uncharacterized enzyme, MaoC, can link the β-oxidation and PHA biosynthetic pathways in E. coli.

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**DISCUSSION**

It has previously been demonstrated that recombinant E. coli impaired in β-oxidation pathway successfully synthesizes MCL-PHAs from fatty acids when equipped with a functional MCL-PHA synthase (15, 22, 24, 25, 27). The elucidated metabolic pathways for the production of MCL-PHA from the intermediates of β-oxidation pathway are shown in Fig. 3. Various enzymes including FadD, FadE, enoyl-CoA hydratase, epimerase and 3-ketoacyl-CoA reductase are involved in the generation of R3HA-CoAs, the substrates for PHA synthase, from fatty acid (Fig. 3).

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Therefore, it was first examined whether the overexpression of the maoC gene could supply PHA precursors from fatty acid. As summarized in Table 3, the coexpression of the maoCEc and phaC2Ps genes in E. coli W3110 successfully allowed MCL-PHA accumulation. Considering that E. coli W3110 harboring only the PHA synthase did not produce PHA from fatty acid, it can be concluded that the overexpression of the maoC gene is necessary for channeling PHA precursors from the β-oxidation pathway. These results also suggest that E. coli MaoC carries out hydration of enoyl-CoAs to make R3HA-CoAs because the PHA synthase accepts only the (R)-form hydroxyacyl-CoAs as substrates.

Recently, another β-oxidation pathway operating under anaerobic conditions using nitrate as a terminal respiratory electron acceptor, which is composed of YfcYX, was found to exist besides aerobic β-oxidation pathway in E. coli (5). Also, it was reported that the deletion of the yfcX gene in fadB mutant E. coli abolished PHA biosynthetic capacity (30). When the fadA and/or fadB genes are deleted, other enzymes such as YfcYX, which are able to use the β-oxidation cycle intermediates as substrates, seem to functionally operate. This phenomenon has been reported by several groups, who showed that various fadA and/or fadB mutant E. coli strains efficiently synthesized MCL-PHAs when a heterologous MCL-PHA synthase gene was introduced (15, 22, 24, 25, 27). A fadB mutant E. coli strain WB101 used in this study synthesized PHA when the Pseudomonas sp. strain 61-3 PHA synthase gene was introduced. Since enoyl-CoAs must be converted to R3HA-CoAs in E. coli, it was reasoned that E. coli may possess another enzyme having enoyl-CoA hydratase activity besides FadB. And, in this study, we have shown by gene knockout study and by enzyme assay that MaoC is the enzyme possessing enoyl-CoA hydratase activity in fadB mutant E. coli. From the results that the deletion of maoCEc gene did not thoroughly abolish the PHA biosynthetic capacity, there should be other enzymes such as YfcX connecting the β-oxidation and PHA biosynthetic pathways.

FIG. 3. Proposed metabolic pathways for PHA biosynthesis in recombinant E. coli strain from fatty acid through β-oxidation pathway. Enoyl-CoA hydratase, epimerase and 3-ketoacyl-CoA or ACP reductase have been employed to supply PHA precursors. The following letters in parentheses represent the indicated enzymes: A, enzymes that have not been elucidated in E. coli; B, amplified enzymes to supply PHA precursors FabG (22, 27, 33), RhlG (22), and PhaJ (9, 23, 34, 35); C, enzymes that are activated when the FadB is inactivated — YfcX (30), MaoC (this study); X, enzymes that should be inactivated to supply PHA precursors from fatty acid (15, 22, 24, 25, 27, 30).
2 and 4 compared with those of supplied fatty acid, was produced by fadB mutant E. coli, even though the key enzyme, FadB, involved in cycling intermediates of 2 and 4 compared with those of supplied fatty acid, was pro-

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