Research Article

Proteome-Level Responses of *Escherichia coli* to Long-Chain Fatty Acids and Use of Fatty Acid Inducible Promoter in Protein Production

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1. INTRODUCTION

Exogenous fatty acids and their derivatives influence a wide variety of cellular processes including fatty acids and phospholipids synthesis, organelle inheritance, vesicle fusion, protein export and modification, enzyme activation or deactivation, cell signaling, membrane permeability, bacterial pathogenesis, and transcriptional control [1, 2]. The process governing the transport of fatty acids from environmental conditions across the membrane is distinct from the transport of hydrophilic substrates such as sugars and amino acids. In a number of cell types, the process of fatty acid transport is inducible and commensurate with the expression of specific sets of proteins [2]. In wild-type *Escherichia coli*, growth on fatty acids requires specific transport system (FadL), acyl-CoA dehydrogenase (FadD), enzymes of the β-oxidation cycle (FadA, FadB, FadE, FadF, FadG, and FadH), and glyoxylate shunt (AceA, AceB, and AceK), and these genes are negatively regulated by a transcriptional factor, FadR. Supply of long-chain fatty acids that contain 12 or more carbons results in the derepression of the genes negatively controlled by FadR but leads to the decreased expression of the genes (e.g., fabA and fabB) activated by FadR, indicating that long-chain acyl-CoA esters are the effector molecules that regulate fatty acids metabolism and thereby mediate inductions [3]. Therefore, *E. coli* cells can grow on minimal medium containing long-chain fatty acids but it cannot grow on short- and medium-chain fatty acids due to no induction of the enzymes associated with fatty acids metabolism. So far, genes involved in fatty acids metabolism (i.e., *fad* regulon) of *E. coli* have been reported at the transcriptional level by biochemical and genetic analyses [3–6]. Therefore, in this study, we looked at the effects of long-chain fatty acids at the translational level of *E. coli*.
Proteomics has changed the way to study cellular physiology. Previously, one or more proteins were chosen as models for understanding local physiological phenomena. Nowadays, proteomic studies allow researchers to identify large members of stimulons, a set of proteins whose amount or synthesis rate changes in response to a certain stimulus, and to obtain information that indicates which specific proteins should be studied further. Comparative proteome profiling under various environmental conditions also reveal new regulatory circuits and the relative abundances of protein sets at the system-wide level. Such analyses of every protein induced or repressed by the stimulus may provide the necessary information to understand a response in the cell. Furthermore, proteome profiles can prove invaluable when used in conjunction with various molecular biological tools including recombinant DNA technology [7]. For example, conditional promoters activated by the specific stimulus, such as the system-wide level. Such analyses of every protein induced or repressed by the stimulus may provide the necessary information to understand a response in the cell. Furthermore, proteome profiles can prove invaluable when used in conjunction with various molecular biological tools including recombinant DNA technology [7]. For example, conditional promoters activated by the specific stimulus, such as the

In this study, proteomic studies that compared global translational differences between E. coli W3110 cells in the presence of glucose and oleic acid (C18) were conducted. The present study has three goals: (i) to identify the stimulon of the oleic acid; (ii) to select target proteins from the stimulon to utilize them as the oleic acid-inducible promoter; and (iii) further to apply it for the production of recombinant proteins in bacteria [8].

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2. MATERIALS AND METHODS

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are shown in Table 1. E. coli XL1-Blue was used as a strain for cloning and maintenance of plasmids. E. coli W3110 was used as a host strain for proteomic studies and the production of a recombinant protein. PCR primers used in this study are listed in Table 2. Primers for the amplification of the promoter regions of cadA and udp genes were designed based on the genome sequence of E. coli K-12 W3110 (AC_000091). The promoter region of cadA gene was amplified by PCR using primers 1 and 2, and was cloned into the EcoRV and EcoRI sites of pTac99A to make pAD99A (Table 1). In fact, pTac99A is a derivative of pTrc99A (Pharmacia Biotech., Uppsala, Sweden), which was constructed by replacing the trc promoter of pTrc99A with the tac promoter from pKK223-3 (Pharmacia Biotech) digested by PvuII and EcoRI [9]. Also, the promoter region of udp gene was amplified by PCR using primers 3 and 4, and was cloned into the EcoRV and EcoRI sites of the high-copy-number plasmid pTac99A to make pUP99A (Table 1). Both promoters were constructed with the ribosome binding sites consisting of the AGGA sequence having an optimal distance length of 8 bases from a start codon [10].

PCR was performed in 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, USA), Taq polymerase and the ABI Prism 377 DNA sequencer (Perkin-Elmer Co., Mass, USA). All DNA manipulations were carried out according to standard procedures [11].

2.2. Cell growth conditions and analytical procedure

Cells were cultivated at 37°C and 250 rpm in 100 mL of Luria-Bertani (LB) medium (10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of NaCl), or R/2 medium plus 10 g/L glucose or 5 g/L oleic acid (Daejung Chemicals & Metals Co., Gyeonggi-do, Korea) as a carbon source. The R/2 medium (pH 6.8) contains per liter: 2 g of (NH4)2HPO4, 6.75 g of KH2PO4, 0.85 g of citric acid, 0.7 g of MgSO4·7H2O, and 5 mL of a trace metal solution. The trace metal solution contains per liter of 5 M HCl: 10 g of FeSO4·7H2O, 2.25 g of ZnSO4·7H2O, 1 g of CuSO4·5H2O, 0.5 g of MnSO4·5H2O, 0.23 g of Na2B4O7·10H2O, 2 g of CaCl2·2H2O, and 0.1 g of (NH4)6Mo7O24. For the cultivation of recombinant E. coli strains, ampicillin (Ap, 50 μg/mL) was added. Cell growth was monitored by measuring the absorbance at 600 nm (OD600; DU Series 600 Spectrophotometer, Beckman, Fullerton, Calif, USA). At an OD600 of 0.7 or 1.2, isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma Chemical Co., St. Louis, MO, USA) was added at a final concentration of 1 mM. For induction by oleic acid, the defined medium supplemented with 10 g/L glucose was changed into the medium plus 5 g/L oleic acid after cells were collected by centrifugation at the same OD600 of 0.7 or 1.2. Then, cells were further cultivated for 5, 10, and 20 hours, and harvested by centrifugation at 3,500 × g for 5 minutes at 4°C. Protein samples were analyzed by electrophoresis on 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [12]. The gels were stained with Coomassie brilliant blue R250 (Bio-Rad, Hercules, Calif, USA), and the protein bands were quantified by a GS-710 Calibrated Imaging Densitometer (Bio-Rad).

2.3. Two-dimensional gel electrophoresis (2DE)

Proteome analysis was performed by 2DE using the IPG-Phor IEF system (GE Healthcare, Chalfont St. Giles, UK) and Protean II xi Cell (Bio-Rad) as described previously [13]. In brief, E. coli W3110 cells grown in the presence of glucose and oleic acid were harvested at the exponential and stationary phases, respectively, by centrifugation for 5 minutes at 3,500 × g and 4°C, and washed four times with low-salt washing buffer. The pellet was then resuspended in 600 μL of a buffer containing 10 mM Tris-HCl (pH 8.0), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.1% (w/v) SDS, and 1% (v/v) cocktail protease inhibitor (Complete Mini EDTA-free; Roche Diagnostics GmbH, Germany). One μL of this sample was mixed with 60 μL of a solution consisting of 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 65 mM DTT, and a trace of bromophenol blue. Proteins (200 μg) quantified by Bradford assay [14] were resuspended in 350 μL of IEF denaturation buffer composed of 8 M urea, 2% (w/v) CHAPS, 20 mM DTT, and 0.8% (v/v) IPG buffer (pH 3–10 NL;
GE Healthcare). The samples were carefully loaded on the IPG strips (18 cm, pH 3–10 NL; GE Healthcare). The loaded IPG strips were rehydrated for 12 hours and focused at 20 °C for 15 minutes at 250 V, followed by 8,000 V until a total of 60,000 V·h was reached. The strips were equilibrated in two equilibration buffers as described previously [15] and then placed on 12% (w/v) SDS-PAGE gels prepared by the standard protocol [12]. Protein spots were visualized using a silver staining kit (GE Healthcare), and the stained gels were scanned by a GS-710 Calibrated Imaging Densitometer (Bio-Rad). ImageMaster 2D Platinum Software (version 5.0; GE Healthcare) was used to identify spots, to match gels, and to quantify spot densities on a volume basis (i.e., integration of spot optical intensity over the spot area).

2.4. Fractionation of outer membrane proteins

Culture broth (3 mL) was centrifuged at 3,500 × g for 5 minutes at 4°C, and the pellet was washed with 1 mL of 10 mM Na2HPO4 buffer (pH 7.2), followed by centrifugation at 3,500 × g for 5 minutes at 4°C. The cell pellet was resuspended in 0.5 mL of 10 mM Na2HPO4 buffer (pH 7.2). Crude extracts of E. coli cells were prepared by five cycles of sonication (each for 15 seconds at 20% of maximum output; High-intensity ultrasonic liquid processors; Sonics & Material Inc., Newtown, Conn, USA). Partially disrupted cells were first removed by centrifugation of sonicated samples at 12,000 × g for 2 minutes at room temperature. Membrane proteins and lipid layers were isolated by centrifugation at 12,000 × g for 30 minutes at 4°C, followed by resuspension in 0.5 mL of 0.5% (w/v) sarcosyl in 10 mM Na2HPO4 buffer (pH 7.2). After incubation at 37°C for 30 minutes, the insoluble pellet containing membrane proteins was obtained by centrifugation at 12,000 × g for 30 minutes at 4°C. Membrane proteins were obtained by washing the insoluble pellet with 10 mM Na2HPO4 buffer (pH 7.2), followed by resuspending in 50 μL of Tris-EDTA buffer (pH 8.0).

2.5. Protein identification by LC-MS/MS analysis

Samples for the MS/MS analysis were prepared as described previously [16]. Briefly, protein spots were excised and destained by incubating in 30 mM potassium ferricyanide and 65 mM sodium thiosulfate for 10 minutes. Gel pieces were washed in Milli-Q water until they became colorless and transparent, and then vacuum-dried. These pieces were proteolysed with 0.02 μg/μL of modified trypsin (Promega, Madison, Wis, USA) in 40 mM ammonium bicarbonate (pH 8.0) as described previously [15]. The Mascot search server

### Table 1: Bacterial strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristics | Reference or source  |
|------------------|-------------------------|---------------------|
| **E. coli strains** |                         |                     |
| XL1-Blue         | recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lac+, F’[proAB lacI q lacZ ΔM15, Tn10 (tetR)] | Stratagene*          |
| W3110            | McrA mcrB IN(rrnD-rrnE)1 | KCTC*               |
| **Plasmids**     |                         |                     |
| pTac99A          | pTrc99A derivative; tac promoter, cloning vehicle; Ap' | Park and Lee [9]     |
| pAD99A           | pTac99A derivative; aldehyde dehydrogenase (aldA) promoter; Ap' | This study           |
| pUP99A           | pTac99A derivative; uridine phosphorylase (udp) promoter; Ap' | This study           |
| pGFpuv           | Ap', lac promoter, gfp   | Clontech*           |
| pAD99GFP         | pAD99A derivative; gfp   | This study           |
| pTac99GFP        | pTac99A derivative; gfp   | This study           |
| pUP99GFP         | pUP99A derivative; gfp   | This study           |

*Stratagene Cloning System (La Jolla, Calif, USA).

**Korean Collection for Type Cultures, (Daejeon, Korea).

*BD Biosciences Clontech (Palo Alto, Calif, USA).

### Table 2: List of primers used in PCR experiments.

| Primer   | Primer sequence | Gene to be amplified | Template            |
|----------|-----------------|----------------------|---------------------|
| Primer 1 | aaaaaccttaactccgacctgacactgactcttt | aldA promoter | E. coli W3110 chromosome |
| Primer 2 | aaaaaccttaactccgacctgacactgactctcc | aldA promoter | E. coli W3110 chromosome |
| Primer 3 | aaaaaccttaactccgacctgacactgactgatttg  | aldA promoter | E. coli W3110 chromosome |
| Primer 4 | aaaaaccttaactccgacctgacactgactgatttg  | aldA promoter | E. coli W3110 chromosome |
| Primer 5 | gaaatctgtgagaaaggaagaacttt | udp promoter | E. coli W3110 chromosome |
| Primer 6 | ccaagcttcggatatgagcctatcc | GFP | pGFpuv |

*Restriction enzyme sites are shown in bold.

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(version 1.8; http://www.matrixscience.com) was used for the identification of protein spots by querying sequence of the tryptic peptide fragments. Reference databases used for the identification of target proteins were UniProt Knowledgebase (Swiss-Prot and TrEMBL; http://kr.expasy.org) and NCBI (http://www.ncbi.nlm.nih.gov).

2.6. Fluorescence microscopy and intensity of GFP

For fluorescence imaging, cells were harvested by centrifugation for 5 minutes at 3,500 x g and 4°C, washed with and resuspended in phosphate-buffered saline (PBS) solution. The samples were mounted on microscopic slide glasses and examined by confocal microscopy (Carl Zeiss, Jena, Germany). Photographs were taken with a Carl Zeiss LSM 410 instrument. Samples were excited by a 364-nm argon laser, and images were filtered by a longpass 505-nm filter. Three-dimensional images were constructed from 5–10 serial images (each 1-Am thick) made by automatic optical sectioning. Fluorescence intensities were measured at 395 nm (excitation) and 509 nm (emission) using the SpectraMax M2 multi-detection system (Molecular Devices, Sunnyvale, Calif, USA), and a 96-well black and clear flat-bottom plate (Coastar, Los Angeles, Calif, USA).

3. RESULTS AND DISCUSSION

3.1. Proteome analysis

To understand physiological changes triggered by the long-chain fatty acid, we analyzed the proteome profiles of E. coli K-12 W3110 grown in the presence of glucose and oleic acid, respectively. The final concentration of cells cultured in oleic acid as a carbon source was 4-fold higher than that of cells grown in glucose, although the former took a longer lag-period for induction of the fad regulon (see Figure 1). Samples of proteome were taken at the exponential and stationary phases in two different media (see Figure 1); when the OD_{600} of E. coli reached 0.57 and 1.25 in the presence of glucose, named G1 and G2, respectively; and when the OD_{600} reached 0.56 and 5 in the presence of oleic acid, named O1 and O2, respectively. The proteome profiles of the four samples, G1, G2, O1, and O2, were analyzed by 2D PAGE using a strip of 3–10 pI range and 12% polyacrylamide gel for subsequent comparisons (see Figure 2). The overall profiles of whole cellular proteins were reproducible. From over 2,000 spots on each 2D gel shown in Figure 2, we identified 92 proteins by comparing with our in-house E. coli proteome database or by conducting LC-MS/MS analysis. Functions and fold changes of individual proteins are shown in Table 3.

The outer membrane proteins were enriched by fractionation, and separated on 12% SDS-PAGE (see Figure 3). Membrane proteins are typically difficult to be resolved in the IEF denaturation buffer used commonly for 2D gels because of their hydrophobic property. The highly abundant porin, OmpF whose expression level was regulated by osmolality [17], was observed at the exponential phase of E. coli in the presence of glucose. As expected, the long-chain fatty acid transporter protein, FadL, was newly synthesized in the presence of oleic acid. This result proves that E. coli requires the specific transport system (fadL) on the growth of fatty acids.

3.2. Identification of the proteins stimulated by oleic acid

To examine the influence of oleic acid on the proteome profile variation, we compared the proteomes obtained from the exponential phase (O1 versus G1) and the stationary phase (O2 versus G2), as shown in Table 3. At the exponential growth phase, the levels of 41 identified proteins were altered in the presence of oleic acid. Among them, 9 proteins including AldA, Cdd, FadA, FadB, MalE, RbsB, Udp, and YccU were newly synthesized in response to oleic acid, while GapA (the fragment), hypothetical protein YfdX, and two unidentified proteins were not detectable. As expected, the levels of proteins involved in fatty acid degradation (FadA and FadB), long-chain fatty acid transport system (FadL), glyoxylate shunt (AceA), and TCA cycle (Mdh, SdhA, SucC, and SucD) were significantly increased to replenish the dicarboxylic acid intermediates consumed in amino acid biosynthesis. Particularly, isocitrate lyase (AceA) in the aceB/AK operon was significantly synthesized by more than five folds in the presence of oleic acid, making it the most abundant protein. Concurrently, there were decreased levels of proteins involved in the biosynthesis of fatty acids (FabD and FabE) and amino acids (AroG, LeuC, and SerC). These results showed that the variation patterns of most proteins identified as a fad regulon were in agreement with their corresponding transcriptional levels previously reported [2–6].

Furthermore, the growth of E. coli on oleic acid involves a significant contribution of the pyrimidine salvage pathway (Cdd and Udp) and specific binding-protein-dependent transport system (MalE and RbsB) because the levels of these proteins highly increased by oleic acid. The salvage pathway of E. coli functions to reutilize free bases and nucleosides.
Table 3: Proteins identified from 2DE.

| Spot no. | Protein name     | Method for identity | Accession no. | pI/Mw*(kDa) | Protein description                                                                 | Fold change\(^b\) | O1/G1 | O2/G2 |
|----------|------------------|---------------------|---------------|-------------|-------------------------------------------------------------------------------------|--------------------|-------|-------|
| 1        | AcnB             | Gel match           | P36683        | 5.24/75.9   | Aconitate hydratase 2                                                               | Δ                  |       |       |
| 2        | AsnS             | Gel match           | P17242        | 5.64/92.8   | Asparaginyl-tRNA synthetase                                                          | —                  |       |       |
| 3        | AceF             | Gel match           | P06959        | 5.01/77.5   | Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex    | \(∇\)              |       |       |
| 4        | DnaK             | Gel match           | P04475        | 4.81/69.6   | Chaperone protein DnaK                                                              | \(∇\)              |       |       |
| 5        | PtsI             | Gel match           | P08839        | 4.78/59.8   | Phosphoenolpyruvate-protein phosphotransferase                                       | \(∇\)              |       | \(3×∇\) |
| 6        | AldA             | MS/MS               | P25553        | 5.07/52.2   | Aldehyde dehydrogenase A 60 kDa chaperonin (GroEL protein)                         | Appeared           |       |       |
| 7        | MopA (GroEL)     | Gel match           | P06139        | 4.85/56.7   | Trigger factor (TF)                                                                 | \(∇\)              |       |       |
| 8        | Tig              | Gel match           | P22257        | 4.83/51.0   | Chaperone protein HtpG (Heat shock protein HtpG)                                     | —                  |       |       |
| 9        | HtpG             | Gel match           | P10413        | 5.06/65.6   | Isocitrate dehydrogenase                                                             | —                  |       |       |
| 10       | AtpD             | Gel match           | P00824        | 4.90/47.7   | Chaperone protein AtpD                                                              | —                  |       |       |
| 11       | Icd (IcdA)       | Gel match           | P08200        | 5.02/46.0   | Elongation factor Tu (EF-Tu)                                                        | —                  |       |       |
| 12       | AceA1            | MS/MS               | P05313        | 5.19/44.1   | Elongation factor Tu (EF-Tu)                                                        | 5×Δ                | 7×Δ   |       |
| 13       | AceA2            | MS/MS               | P08859        | 5.30/50.6   | Isocitrate lyase fragment                                                            | 6×Δ                | 7×Δ   |       |
| 14       | GlnA             | Gel match           | P06711        | 5.25/53.8   | Glutamine synthetase                                                                | —                  |       |       |
| 15       | IlvC             | Gel match           | P05793        | 5.26/52.0   | Ketol-acid reductoisomerase                                                         | —                  |       |       |
| 16       | GlpK             | Gel match           | P08324        | 5.34/46.5   | Glycerol kinase (Glycerokinase)                                                      | —                  |       |       |
| 17       | Eno              | Gel match           | P02990        | 5.32/44.6   | Elongation factor Tu (EF-Tu)                                                        | —                  |       |       |
| 18       | FabD (TfpA)      | Gel match           | P25715        | 5.37/44.8   | Malonyl CoA-acyl carrier protein transacylase                                        | \(∇\)              |       |       |
| 19       | LeuC             | Gel match           | P30127        | 5.42/44.4   | 3-isopropylmalate dehydrogenase large subunit                                       | \(∇\)              |       |       |
| 20       | FadB             | MS/MS               | P21177        | 5.84/79.5   | Fatty oxidation complex alpha subunit                                               | Appeared           |       |       |
| 21       | SdhA             | Gel match           | P10444        | 5.74/63.7   | Succinate dehydrogenase flavoprotein subunit                                        | —                  |       |       |
| 22       | OppA             | Gel match           | P23843        | 5.93/56.1   | Periplasmic oligopeptide-binding protein                                             | \(∇\)              |       |       |
| 23       | TrpD             | Gel match           | P00904        | 6.08/55.9   | Anthranilate synthase component II; Anthranilate                                     | —                  |       |       |
| 24       | GuaB (GuaR)      | Gel match           | P06981        | 6.01/55.0   | Inosine-\(5’\)-monophosphate dehydrogenase                                         | \(∇\)              |       |       |
| 25       | AtpA             | Gel match           | P00822        | 5.84/53.1   | ATP synthase alpha chain                                                             | —                  |       | Δ     |
| 26       | DppA             | Gel match           | P23847        | 5.69/52.1   | Periplasmic dipeptide transport protein                                              | —                  |       |       |
| 27       | GlyA             | Gel match           | P00477        | 6.04/45.9   | Serine hydroxymethyltransferase (Serine methylase)                                  | —                  |       |       |
| 28       | CarA (PyrA)      | Gel match           | P00907        | 5.91/44.0   | Carbamoyl-phosphate synthase small chain                                            | —                  |       |       |
| 29       | Unknown          | MS/MS               | —             | —            | Disappeared                                                                       | —                  |       |       |
| Spot no. | Protein name | Method for identity | Accession no. | pI/Mw<sup>a</sup> (kDa) | Protein description | Fold change<sup>b</sup> | O1/G1 | O2/G2 |
|---------|--------------|---------------------|---------------|--------------------------|---------------------|----------------------|-------|-------|
| 30      | Unknown      | MS/MS               | —             | —                        | —                   | Disappeared          | —     | —     |
| 31      | FadA         | MS/MS               | P21151        | 6.31/40.9                | Fatty oxidation complex beta subunit | Appeared            | —     | —     |
| 32      | Fba          | Gel match           | P11604        | 5.55/40.6                | Fructose-bisphosphate aldolase class II | —                  | —     | —     |
| 33      | SerC (PdxF)  | Gel match           | P23721        | 5.34/40.2                | Phosphoserine aminotransferase | ∇      | ∇     |
| 34      | SucC         | Gel match           | P07460        | 5.30/42.3                | Succinyl-CoA synthetase beta chain | Δ      | Δ     |
| 35      | LivJ         | MS/MS               | P02917        | 5.28/41.9                | Leu/Ile/Val-binding protein | —     | —     |
| 36      | Pgk          | Gel match           | P11665        | 5.07/41.9                | Phosphoglycerate kinase | —     | ∇     |
| 37      | MalE         | MS/MS               | P02928        | 5.08/41.1                | Malate-binding periplasmic protein | Appeared           | —     | —     |
| 38      | LivK         | Gel match           | P04816        | 5.00/41.4                | Leucine-specific binding protein | —     | —     |
| 39      | RfaD (HtrM)  | Gel match           | P17963        | 4.85/36.8                | ADP-L-glycero-D-manno-heptose-6-epimerase | —     | —     |
| 40      | PotD         | Gel match           | P23861        | 4.77/35.8                | Spermidine/putrescine-binding periplasmic protein | —     | —     |
| 41      | TalB         | Gel match           | P30148        | 5.01/35.8                | Transaldolase B | —     | ∇     |
| 42      | Tsf (EF-Ts)  | Gel match           | P02997        | 5.15/33.6                | Elongation factor Tsf (EF-Ts) | —     | ∇     |
| 43      | Mdh          | MS/MS               | P06994        | 5.55/35.5                | Malate dehydrogenase | Δ     | —     |
| 44      | CysK         | Gel match           | P11096        | 5.81/36.0                | Cysteine synthase A | —     | —     |
| 45      | ManX (PtsL)  | Gel match           | P08186        | 5.17/26.1                | PTS system, mannose-specific IIAB component | ∇     | —     |
| 46      | Unknown      | MS/MS               | —             | —                        | 2×∇ 3×∇ | —     | —     |
| 47      | AroG         | Gel match           | P00886        | 6.12/39.4                | Phospho-2-dehydro-3-deoxyheptonate aldolase | 2×∇  ∇ | ∇     |
| 48      | Sbp          | Gel match           | P06997        | 6.49/49.5                | Sulfate-binding protein | 2×∇  ∇ | ∇     |
| 49      | GapA         | Gel match           | P06977        | 6.58/36.3                | Glyceraldehyde 3-phosphate dehydrogenase A | —     | —     |
| 50      | PyrB         | Gel match           | P00479        | 6.13/35.3                | Aspartate carbamoyltransferase catalytic chain | —     | —     |
| 51      | FkpA         | Gel match           | P45523        | 7.08/33.2                | FKB-type peptidyl-prolyl cis-trans isomerase FkpA | —     | —     |
| 52      | SucD         | MS/MS               | P07459        | 6.31/29.6                | Succinyl-CoA synthetase alpha chain | Δ     | Δ     |
| 53      | GapA         | MS/MS               | P06977        | 6.58/23.0                | No. 49 fragment | Disappeared | —     | —     |
| 54      | GlnH         | MS/MS               | P10344        | 6.87/24.9                | Glutamine-binding periplasmic protein | —     | —     |
| 55      | SodA         | Gel match           | P00448        | 6.44/22.9                | Superoxide dismutase [Mn] (MnSOD) | —     | —     |
| 56      | RbsB         | MS/MS               | P02925        | 5.92/29.1                | D-ribose-binding periplasmic protein | Appeared | —     |
| 57      | Udp          | Gel match           | P12758        | 5.86/27.9                | Uridine phosphorylase (UDRPase) | Appeared | —     |
| 58      | YadK         | Gel match           | P37016        | 5.55/28.4                | Protein YadK | —     | —     |
| 59      | TpiA (Tpi)   | Gel match           | P04790        | 5.57/26.9                | Triosephosphate isomerase | —     | —     |
| Spot no. | Protein name | Method for identity | Accession no. | pI/Mw* (kDa) | Protein description | Fold changeb | O1/G1 | O2/G2 |
|----------|--------------|---------------------|---------------|--------------|---------------------|-------------|-------|-------|
| 60       | Cdd          | MS/MS               | P13652        | 5.08/31.5    | Cytidine deaminase  | Appeared    | Appeared | Appeared |
| 61       | TrpA         | Gel match           | P00928        | 5.30/28.7    | Tryptophan synthase alpha chain | —           | —       | —       |
| 62       | SspA (Ssp)   | Gel match           | P05838        | 5.24/26.6    | Stringent starvation protein A | —           | —       | —       |
| 63       | HisJ         | Gel match           | P39182        | 5.05/28.6    | Histidine-binding periplasmic protein | —           | —       | —       |
| 64       | FliY         | Gel match           | P39174        | 5.01/26.2    | Cystine-binding periplasmic protein | ∇           | 3×∇     | —       |
| 65       | HdhA (HsdH)  | Gel match           | P25529        | 5.17/25.0    | 7-alpha-hydroxysteroid dehydrogenase | ∇           | —       | —       |
| 66       | Upp (UraP)   | Gel match           | P25532        | 5.29/23.8    | Uracl phosphoribosyltransferase | —           | —       | —       |
| 67       | GrpE         | Gel match           | P09372        | 4.68/25.5    | GrpE protein (HSP-70 cofactor) | —           | 2×∇     | —       |
| 68       | AccB (FabE)  | Gel match           | P02905        | 4.57/22.0    | Biotin carboxyl carrier protein of acetyl-CoA carboxylase | 2×∇         | 2×∇     | —       |
| 69       | YfdX         | MS/MS               | P76520        | 5.38/23.0    | Protein yfdX         | Disappeared  | Disappeared | —       |
| 70       | AhpC         | Gel match           | P26427        | 5.01/21.5    | Alkyl hydroperoxide reductase C22 protein | ∇           | —       | ∇       |
| 71       | Crr          | Gel match           | P08837        | 4.57/20.0    | PTS system, glucose-specific IIa component | —           | —       | —       |
| 72       | DksA         | Gel match           | P18274        | 4.90/18.7    | DnaK suppressor protein | —           | ∇       | —       |
| 73       | AroK         | Gel match           | P24167        | 5.30/17.9    | Shikimate kinase I | —           | —       | —       |
| 74       | SodB         | Gel match           | P09157        | 5.53/22.1    | Superoxide dismutase [Fe] | 2×∇         | ∇       | —       |
| 75       | PpiB         | Gel match           | P23869        | 5.51/17.7    | Peptidyl-prolyl cis-trans isomerase B | —           | ∇       | —       |
| 76       | RplL         | Gel match           | P02418        | 6.20/19.8    | 50S ribosomal protein L9 | ∇           | ∇       | —       |
| 77       | YbdQ         | Gel match           | P39177        | 6.08/15.5    | Unknown protein from 2D-page | —           | —       | —       |
| 78       | RbfA         | Gel match           | P09170        | 6.00/15.6    | Ribosome-binding factor A | —           | —       | —       |
| 79       | RplU         | Gel match           | P02422        | 6.71/10.3    | 50S ribosomal protein L21 | 2×∇         | 3×∇     | —       |
| 80       | Hns          | Gel match           | P08936        | 5.45/15.6    | DNA-binding protein H-NS (Histone-like protein HLP-II) | —           | Δ       | —       |
| 81       | Ndk          | Gel match           | P24233        | 5.59/15.2    | Nucleoside diphosphate kinase (NDP kinase) | —           | —       | —       |
| 82       | AtpC         | Gel match           | P00832        | 5.48/14.8    | ATP synthase epsilon chain | —           | —       | —       |
| 83       | RpsF         | Gel match           | P02358        | 5.15/15.8    | 30S ribosomal protein S6 | —           | —       | —       |
| 84       | Bcp          | Gel match           | P23480        | 5.02/15.8    | Bacterioferritin comigratory protein | ∇           | 2×∇     | —       |
| 85       | GreA         | Gel match           | P21346        | 4.68/15.9    | Transcription elongation factor GreA | 2×∇         | Disappeared | —       |
| 86       | GroES (MopB) | Gel match           | P05380        | 5.15/15.6    | 10 kDa chaperonin (GroES protein) | —           | —       | —       |
| 87       | YifD         | MS/MS               | P33633        | 5.09/14.3    | Protein YifD | —           | —       | —       |
produced intracellularly from nucleotide turnover [18]. Also, the pyrimidine salvage pathway has been reported to recycle the pentose moieties of exogenous nucleosides to use them as carbon and energy sources and the amino groups of cytosine compounds as a nitrogen source. The D-ribose-binding periplasmic protein, RbsB in the rbsACBK operon, mediates the entry of D-ribose across the cell membrane in the form of D-ribose 5-phosphate, which is an intermediate of the pentose phosphate cycle [19]. Therefore, long-chain fatty acids seem to influence the status of the pyrimidine salvage pathway and its associated transport system.

Interestingly, aldehyde dehydrogenase (AldA), which oxidizes diverse aldehydes throughout the cellular metabolism, received a special attention in this study because of its possible applications in gene expression system with oleic acid as an inducer. It has been reported that the expression of aldA gene was induced on growth on fucose, rhamnose, arabinose, glutamate, 2-oxoglutarate during aerobic condition, while that is repressed by glucose [20]. Our observation found in this study demonstrated that oleic acid is another inducer of the aldA gene. Its application as an inducible promoter is demonstrated in the next two sections.

At the stationary phase, the levels of 45 identified proteins were altered in response to oleic acid (Table 3). Sixteen proteins including AceA, AcmB, AldA, AtxA, Cdd, FadA, FadB, FadL, Hns, Icd, MalE, RbsB, SucC, SucD, Udp, and YccU were significantly increased or newly synthesized, while 29 proteins were reduced or disappeared on 2D gels. Although the variations of proteins at the stationary phase were more complex, most proteins with altered levels on 2D gels at this phase showed patterns similar to those at the exponential growth phase.

### 3.3. Construction of the expression system with oleic acid-inducible promoters

Among proteins highly inducible by oleic acid, we selected two target proteins, AldA and Udp according to the following two criteria for their utilization as promoters: (i) they are only induced in the presence of oleic acid to be strictly controlled; (ii) they are strongly and highly expressed for the enhanced bioproducts production. AldA and Udp were synthesized in response to oleic acid with relatively high abundance from the exponential to stationary phases, and were not synthesized in the presence of glucose, suggesting that the native promoters of these proteins could be used as an oleic acid-inducible promoter in E. coli W3110.

For the construction of the expression systems controlled by aldA or udp promoter, the promoter regions of these genes were amplified as described in Section 2. The representative schematic plasmid map under the control of aldA promoter is illustrated in Figure 4. This is a high-copy-number plasmid with replication origin of pBR322 (ATCC 37017).

### 3.4. Comparison between the expression efficiency of oleic acid- and IPTG-inducible promoters

Various cultivation strategies employing different host strains and expression systems have been employed for the production of recombinant proteins [10, 21]. One of the most popular approaches is the use of different promoters to regulate expression levels [10]. In E. coli, many inducible promoters have been developed, which can be induced by various mechanisms such as temperature upshifting, pH fluctuation, nutrient starvation, and addition of chemical inducers. Among these inducible systems, T7 or lac-based promoters (lac, trc, lacUV5-T7 hybrid, etc.), which can be effectively induced by the addition of IPTG, are the most frequently used ones.

In order to evaluate the effectiveness of oleic acid-inducible promoters discovered in this study, we chose the IPTG-inducible tac promoter as a control. Green fluorescent protein (GFP) from the jellyfish Aequorea Victoria was employed as a model recombinant protein to examine its expression under the control of aldA or udp promoter.

For the induction of GFP by oleic acid, the defined medium supplemented with glucose was transferred into oleic acid medium at the OD_{600} of 0.7 or 1.2. For the control, cells harboring the plasmid containing tac promoter were added with 1 mM IPTG at the same values of OD_{600} in the defined medium supplemented with glucose. After induction by IPTG or oleic acid, cells were further cultured, harvested at each time, and analyzed by 12% SDS-PAGE (see Figure 5). When GFP is induced at the OD_{600} of 1.2 in recombinant E. coli W3110 harboring pTac99GFP, pAD99GFP, and pUP99GFP, its contents were approximately 27%, 42%, and 25% of the total proteins at 10 hours, and 28%, 50%, and 25% at 20 hours, respectively. The GFP content induced by aldA promoter was

| Table 3: Continued. |
|---------------------|
| Spot no. | Protein name | Method for identity | Accession no. | pI/ MW (kDa) | Protein description | Fold change<sup>b</sup> |
| 88 | UspA | Gel match | P28242 | 5.14/15.1 | Universal stress protein A | — |
| 89 | YigF | Gel match | P39330 | 5.29/13.0 | Protein YigF | ▼ |
| 90 | TrxA (TsnC) | Gel match | P00274 | 4.67/11.5 | Thioredoxin 1 | — |
| 91 | HdeB | Gel match | P26605 | 4.85/11.2 | Protein HdeB (10K-L protein) | — |
| 92 | YccU | MS/MS | P75874 | 6.72/14.7 | Protein YccU; Predicted CoA-binding protein | Appeared |

<sup>a</sup>Unit of the molecular weight (MW) is kDa.<br><sup>b</sup>Fold change: 0 ~ 0.3-fold, ▼; 0.3 ~ 0.5-fold, 2× ▼; 0.5 ~ 0.6-fold, ▼; 1.5-fold, △; 2-fold, 2× △; fold change, fold number △.
Figure 2: The 2DE maps of *E. coli* W3110 cells at the exponential (left panels; A, C) and stationary phases (right panels; B, D) in the presence of glucose (A, B) and oleic acid (C, D), respectively. Identified proteins shown in numbers are listed in Table 3. Boxes further highlight specific corresponding regions of the 2D gel images, which are compared at higher resolution in the bottom of (E). Arrow lines indicate individual spots of AldA and Udp.
about 2-fold higher than that obtained by IPTG-inducible tac or udp promoter. Under the IPTG-inducible promoter, the expression of GFP was low even in LB medium compared to the oleic acid-inducible aldA promoter (data not shown). Additionally, the final cell concentration of recombinant E. coli W3110 cells in the presence of oleic acid was 4-fold higher than that of recombinant E. coli cultured in glucose as a carbon source. This result was further confirmed by fluorescence intensity measurements and confocal microscopy (see Figure 6). Strong fluorescence was uniformly detected in recombinant E. coli cells under the control of aldA promoter. The fluorescence intensity of GFP obtained from W3110 harboring pAD99GFP was more than 30-fold higher than that obtained from W3110 harboring pTac99GFP, indicating that the aldA promoter efficiently enhances recombinant protein production compared to the tac promoter. Since the aldA promoter was not activated by glucose, GFP was not produced in the presence of glucose in accordance with the proteome profiles. GFP was only produced under the aldA promoter along with the supply of exogenous oleic acid. However, the tac promoter was not tightly controlled, leaking the recombinant protein even without IPTG induction. These results manifest that the aldA promoter is very efficient for the production of recombinant proteins in E. coli as an inducible promoter. The maximum productivity can be achieved when the growth and production phases are separated as conducted in this study. Separation of the two phases is often achieved by delaying induction time until the cell density reaches a suitable value.

Until now, the IPTG-inducible promoters tac or trc have been widely used for basic research. However, the use of IPTG for the large-scale production of human therapeutic proteins is undesirable because of its toxicity and relatively high cost; the high concentration of IPTG can inhibit cell growth and recombinant protein production [22, 23]. Therefore, determination of optimal induction time point as well as the inducer concentration is crucial to increase the overall productivity of recombinant protein. In this regard, there have been significant efforts to overcome such problems by using different inducible or even constitutive expression systems [24]. Thus, the aldA promoter found in this study can be effectively used for the enhanced production of recombinant proteins with the aforementioned problems largely resolved.

In summary, the aldA promoter satisfies requirements for its utilization as a promoter. First, it is tightly controllable with an appropriate inducer, oleic acid in this case. Tight regulation of the promoter is essential for the synthesis of proteins which may be detrimental to the host cell. For example, the toxic rotavirus VP7 protein effectively kills cells, and must be produced under tightly regulated conditions [25]. Second, its expression is strong and long lasting, resulting in the accumulation of the target protein constituting up to 50% of the total cellular proteins. The third important characteristic of aldA promoter is its inducibility in a cost-effective manner by using exogenous oleic acid as an inducer.

4. CONCLUSION

Proteome analysis of the cells with focus on proteins induced or repressed by the stimulus provides clues to the understanding of cellular responses. This study revealed that 52 proteins showed significantly altered levels in E. coli grown with oleic acid compared to the glucose. Based on the resulting proteome profiles, the promoter of aldA gene was...
Figure 5: The effect of the recombinant protein production by oleic acid-inducible promoter in recombinant E. coli W3110. The cells are induced by exchanging medium supplemented with glucose into the one with oleic acid at the OD$_{600}$ of 0.7 (a) or 1.2 (b). For the control, cells harboring the plasmid containing tac promoter were added with 1 mM IPTG at the same values of OD$_{600}$ in the defined medium supplemented with glucose. After induction by IPTG or oleic acid, cells were further cultured for 5, 10, 20 hours, and harvested for 12% (w/v) SDS-PAGE. The arrows indicate the green fluorescent protein (GFP; 26.9 kDa). Size markers (in kDa) are also indicated.

Figure 6: The fluorescence intensities (a) and confocal microscopic images (b) of E. coli W3110 cells by induction with IPTG (middle panels) or oleic acid (bottom panels). As a control, the E. coli strain without plasmid is also shown (top panels). Shown in (b) are immunofluorescence micrographs (left panels), differential interference micrographs (middle panels), and merged images (right panels) of wild-type E. coli W3110 and its recombinant cells harboring pTac99GFP and pAD99GFP.
found to be strongly activated by oleic acid and subsequently to be demonstrated useful as an inducible promoter for the enhanced production of desirable targets. Thus, this study demonstrates that E. coli proteome profiles not only provide invaluable information for physiological status of the organism under specific conditions but also propose its biotechnological applications.

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