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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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In *Escherichia coli*, a long-chain acyl-CoA is a regulatory signal that modulates gene expression through its binding to a transcription factor FadR. In this study, comparative proteomic analysis of *E. coli* in the presence of glucose and oleic acid was performed to understand cell physiology in response to oleic acid. Among total of 52 proteins showing altered expression levels with oleic acid presence, 9 proteins including AldA, Cdd, FadA, FadB, FadL, MalE, RbsB, Udp, and YccU were newly synthesized. Among the genes that were induced by oleic acid, the promoter of the *aldA* gene was used for the production of a green fluorescent protein (GFP). Analysis of fluorescence intensities and confocal microscopic images revealed that soluble GFP was highly expressed under the control of the *aldA* promoter. These results suggest that proteomics is playing an important role not only in biological research but also in various biotechnological applications.

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 stationary phase, pH, temperature, and nutrient limitation have been used for efficient production of heterologous proteins in bacteria [8].

In this study, proteomic studies that compared global translational differences between *E. coli* W3110 cells in the presence of glucose and oleic acid (C_{18}) 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 or other biotechnological systems.

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 forcloning 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 *aldA* and *udp* genes were designed based on the genome sequence of *E. coli* K-12 W3110 (AC_000091). The promoter region of *aldA* gene was amplified by PCR using primers 1 and 2, and was cloned into the *Eco*RV and *Eco*RI 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 *Pvu*II and *Eco*RI [9]. Also, the promoter region of *udp* gene was amplified by PCR using primers 3 and 4, and was cloned into the *Eco*RV and *Eco*RI 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 $(NH_4)_2HPO_4$, 6.75 g of KH_2PO_4, 0.85 g of citric acid, 0.7 g of $MgSO_4 \cdot 7H_2O$, and 5 mL of a trace metal solution. The trace metal solution contains per liter of 5 M HCl : 10 g of $FeSO_4 \cdot 7H_2O$, 2.25 g of $ZnSO_4 \cdot 7H_2O$, 1 g of $CuSO_4 \cdot 5H_2O$, 0.5 g of $MnSO_4 \cdot 5H_2O$, 0.23 g of $Na_2B_4O_7 \cdot 10H_2O$, 2 g of $CaCl_2 \cdot 2H_2O$, and 0.1 g of $(NH_4)_6MO_7O_{24}$. 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 (OD_{600}; DU Series 600 Spectrophotometer, Beckman, Fullerton, Calif, USA). At an OD_{600} 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 OD_{600} 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 IPGphor 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 $MgCl_2$, 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;

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, suppE44, relA1, l⁻, lac⁻, F'[proAB lacI^q lacZ ΔM15, Tn10 (tet)^r]*	Stratagene[a]
W3110	*F⁻ mcrA mcrB IN(rrnD⁻ rrnE)1λ⁻*	KCTC[b]
Plasmids		
pTac99A	pTrc99A derivative; *tac* promoter, cloning vehicle; Ap^r	Park and Lee [9]
pAD99A	pTac99A derivative; aldehyde dehydrogenase (*aldA*) promoter; Ap^r	This study
pUP99A	pTac99A derivative; uridine phosphorylase (*udp*) promoter; Ap^r	This study
pGFPuv	Ap^r, *lac* promoter, *gfp*	Clontech[c]
pAD99GFP	pAD99A derivative; *gfp*	This study
pTac99GFP	pTac99A derivative; *gfp*	This study
pUP99GFP	pUP99A derivative; *gfp*	This study

[a] Stratagene Cloning System (La Jolla, Calif, USA).
[b] Korean Collection for Type Cultures, (Daejeon, Korea).
[c] BD Biosciences Clontech (Palo Alto, Calif, USA).

Table 2: List of primers used in PCR experiments.

Primer	Primer sequence[a]	Gene to be amplified	Template
Primer 1	aaaaccgtt**gatatc**tttgcaaacgggcatgactcctgactttt	*aldA* promoter	*E. coli* W3110 chromosome
Primer 2	aaaaccgtt**gaattc**ctcctgtgatttatatgtttgttttc		
Primer 3	aaaaccgtt**gatatc**tgcagaatgaagggtgatttatgtgatttg	*udp* promoter	*E. coli* W3110 chromosome
Primer 4	aaaaccgtt**gaattc**ctcctctgtgaatcggtttagtcaga		
Primer 5	g**gaattc**atgagtaaaggagaagaacttt	GFP	pGFPuv
Primer 6	ccc**aagctt**ttatttgatgagctcatcc		

[a] Restriction enzyme sites are shown in bold.

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 Na_2HPO_4 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 Na_2HPO_4 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 Na_2HPO_4 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 Na_2HPO_4 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 for overnight at 37°C. Tryptic peptides (10 μL aliquots) were analyzed by a nano-LC/MS system consisting of an Ultimate HPLC system (LC Packings, Amsterdam, Netherlands) and a quadrupole-time-of-flight (Q-TOF) MS (Micromass, Manchester, UK) equipped with a nano-ESI source as described previously [15]. The MASCOT search server

(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 × 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 p*I* 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 osmolarity [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

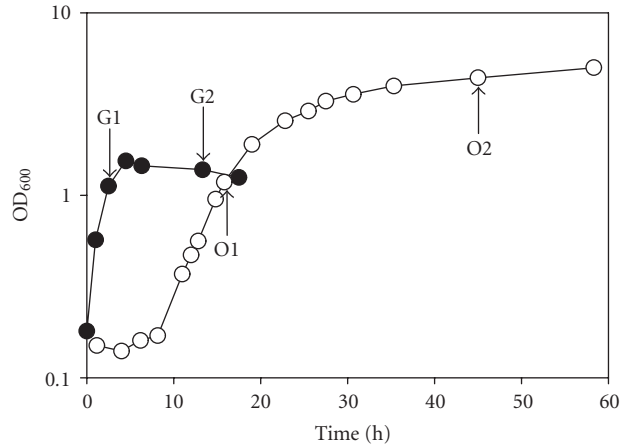

Figure 1: Time profiles of the concentrations of *E. coli* cells. The cell densities (OD_{600}) of *E. coli* W3110 in the presence of glucose (•) or oleic acid (◦) are shown. Gl, G2, O1, and O2 are the sampling points for proteome analyses.

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, FadL, 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 *aceBAK* 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.	p^I/Mw[a] (kDa)	Protein description	Fold change[b]	
						O1/G1	O2/G2
1	AcnB	Gel match	P36683	5.24/75.9	Aconitate hydratase 2	Δ	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	Appeared	Appeared
7	MopA (GroEL)	Gel match	P06139	4.85/56.7	60 kDa chaperonin (GroEL protein)	∇	∇
8	Tig	Gel match	P22257	4.83/51.0	Trigger factor (TF)	—	—
9	HtpG	Gel match	P10413	5.06/65.6	Chaperone protein HtpG (Heat shock protein HtpG)	—	—
10	AtpD	Gel match	P00824	4.90/47.7	ATP synthase beta chain	—	—
11	Icd (IcdA)	Gel match	P08200	5.02/46.0	Isocitrate dehydrogenase	—	Δ
12	AceA1	MS/MS	P05313	5.19/44.1 5.12/44.1	Isocitrate lyase	5×Δ	7×Δ
	AceA2	MS/MS		5.01/33.8	Isocitrate lyase fragment	6×Δ	7×Δ
13	GlnA	Gel match	P06711	5.25/53.8	Glutamine synthetase	—	—
14	IlvC	Gel match	P05793	5.26/52.0	Ketol-acid reductoisomerase	—	—
15	GlpK	Gel match	P08859	5.30/50.6	Glycerol kinase (Glycerokinase)	—	—
16	Eno	Gel match	P08324	5.34/46.5 5.29/46.2	Enolase (2-phosphoglycerate dehydratase)	—	∇
17	TufA (EF-Tu)	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 5.95/51.6	3-isopropylmalate dehydratase large subunit	∇	—
20	FadB	MS/MS	P21177	5.84/79.5	Fatty oxidation complex alpha subunit	Appeared	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 5.94/46.1	Serine hydroxymethyltransferase (Serine methylase)	—	—
28	CarA (PyrA)	Gel match	P00907	5.91/44.0	Carbamoyl-phosphate synthase small chain	—	—
29	Unknown	MS/MS	—	—	—	Disappeared	—

Table 3: Continued.

Spot no.	Protein name	Method for identity	Accession no.	p^I/Mw[a] (kDa)	Protein description	Fold change[b]	
						O1/G1	O2/G2
30	Unknown	MS/MS	—	—	—	Disappeared	Disappeared
31	FadA	MS/MS	P21151	6.31/40.9	Fatty oxidation complex beta subunit	Appeared	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 5.02/41.7	Phosphoglycerate kinase	—	∇
37	MalE	MS/MS	P02928	5.08/41.1	Maltose-binding periplasmic protein	Appeared	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 Ts (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	FKBP-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	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	Appeared
57	Udp	Gel match	P12758	5.86/27.9	Uridine phosphorylase (UDRPase)	Appeared	Appeared
58	YadK	Gel match	P37016	5.55/28.4	Protein YadK	—	—
59	TpiA (Tpi)	Gel match	P04790	5.57/26.9	Triosephosphate isomerase	—	—

Table 3: Continued.

Spot no.	Protein name	Method for identity	Accession no.	p[I]/Mw[a] (kDa)	Protein description	Fold change[b]	
						O1/G1	O2/G2
60	Cdd	MS/MS	P13652	5.08/31.5	Cytidine deaminase	Appeared	Appeared
		MS/MS		5.42/31.5		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 5.11/25.8	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	Uracil 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 4.68/18.9	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	RplI	Gel match	P02418	6.20/19.8 6.17/15.7	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	5.31/15.8 5.15/15.8 5.26/15.8	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	YfiD	MS/MS	P33633	5.09/14.3	Protein YfiD	—	—

Table 3: Continued.

Spot no.	Protein name	Method for identity	Accession no.	p^I/Mw[a] (kDa)	Protein description	Fold change[b]	
						O1/G1	O2/G2
88	UspA	Gel match	P28242	5.14/15.1	Universal stress protein A	—	—
89	YjgF	Gel match	P39330	5.29/13.0	Protein YjgF	∇	∇
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)	—	3×∇
92	YccU	MS/MS	P75874	6.72/14.7	Protein YccU; Predicted CoA-binding protein	Appeared	Appeared

[a] Unit of the molecular weight (MW) is kDa.
[b] Fold change: 0 ∼0.3-fold, 3×∇; 0.3 ∼0.5-fold, 2×∇; 0.5 ∼0.6-fold, ∇; 1.5-fold, Δ; 2-fold, 2×Δ; fold change, fold number×Δ.

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 oxidize 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, or 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, AcnB, AldA, AtpA, 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 (*tac*, *trc*, *lac*, *lac*UV5-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

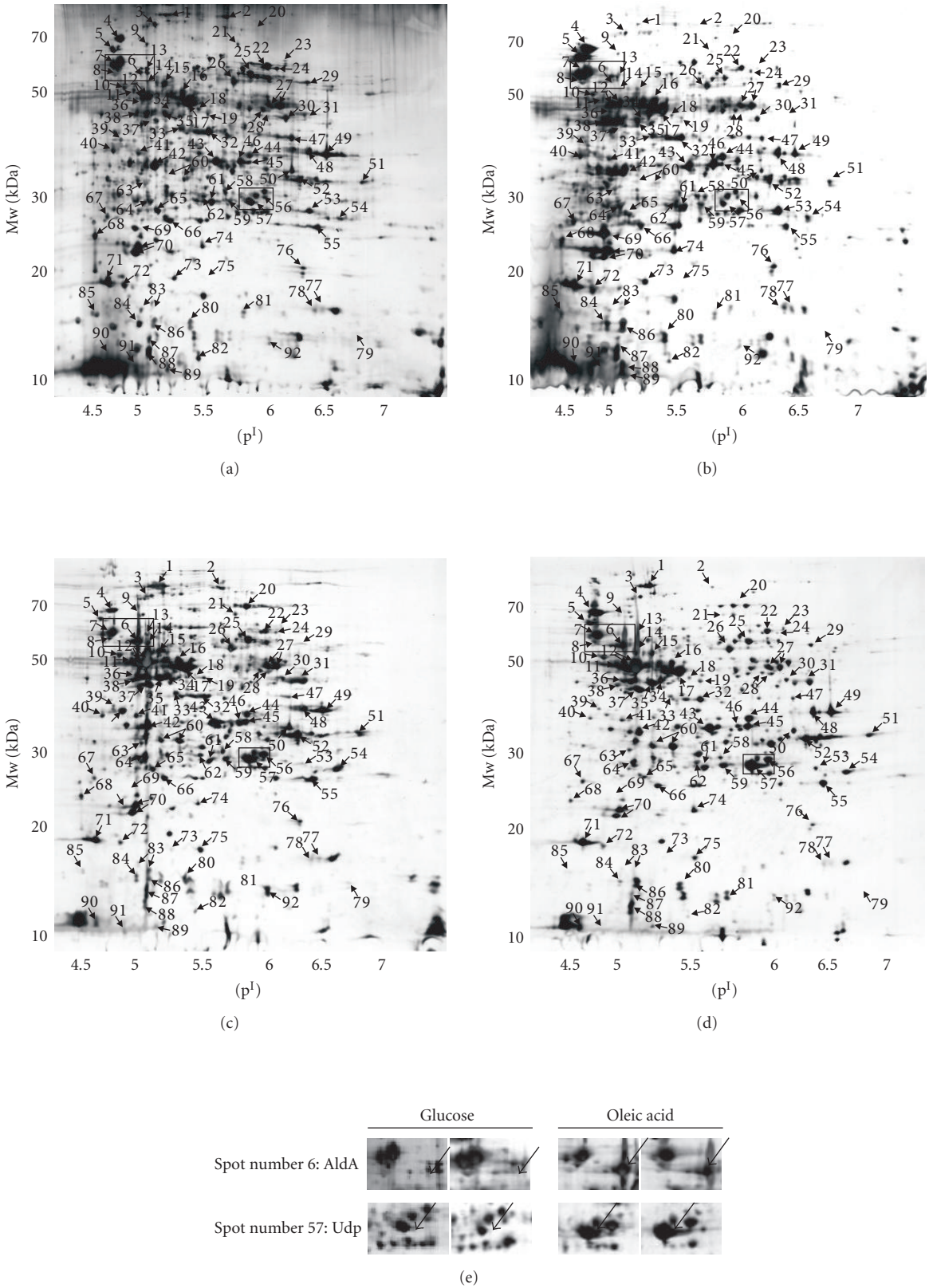

Figure 2: The 2DE maps of *E. coli* W3110 cells at the exponential (left panels; A, C) and stationary phases (right panels; B, D) in the presence of glucose (A, B) and oleic acid (C, D), respectively. Identified proteins shown in numbers are listed in Table 3. Boxes further highlight specific corresponding 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.

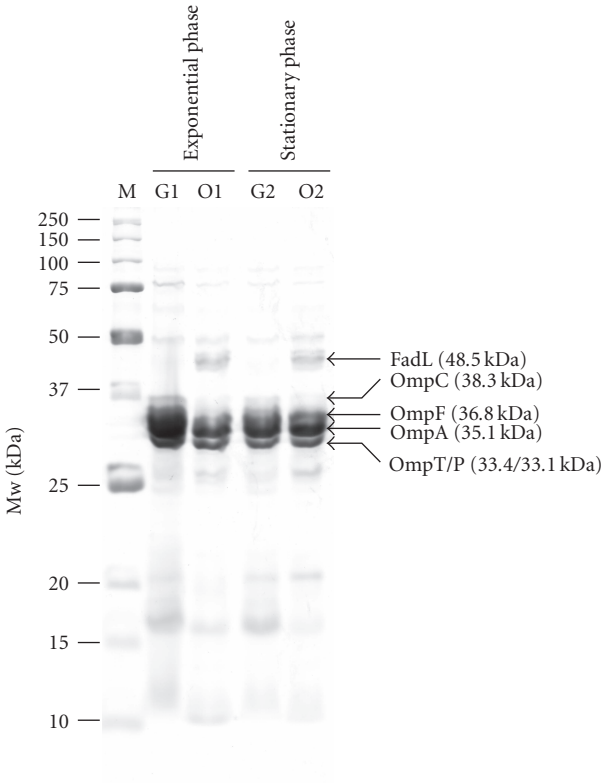

Figure 3: SDS-PAGE of the outer membrane proteins taken from samples of proteome. Identified proteins are shown on the right side. Size markers (in kDa) are indicated on the left.

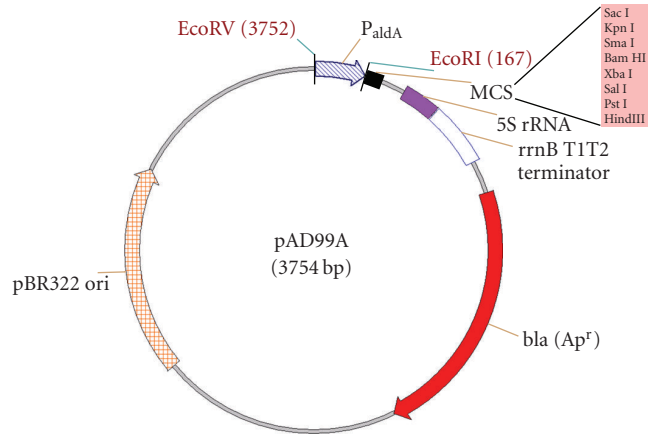

Figure 4: Map of plasmid pAD99A. Its characteristics include pBR322 ori, origin of replication of pBR322; *bla*, ampicillin-resistance gene; P_{aldA}, *aldA* promoter; a target gene to be inserted in multiple cloning sites (MCSs).

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

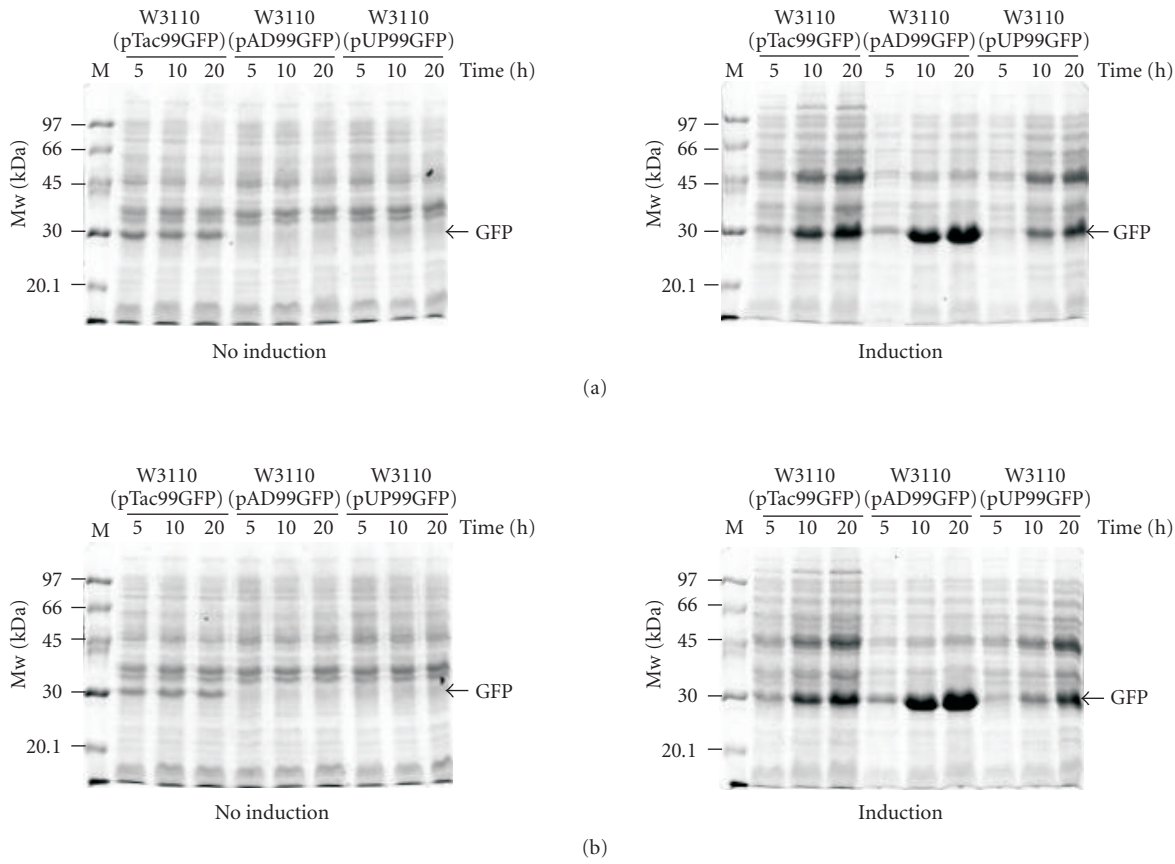

Figure 5: The effect of the recombinant protein production by oleic acid-inducible promoter in recombinant *E. coli* W3110. The cells are induced by exchanging medium supplemented with glucose into the one with oleic acid at the OD_{600} of 0.7 (a) or 1.2 (b). For the control, cells harboring the plasmid 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.

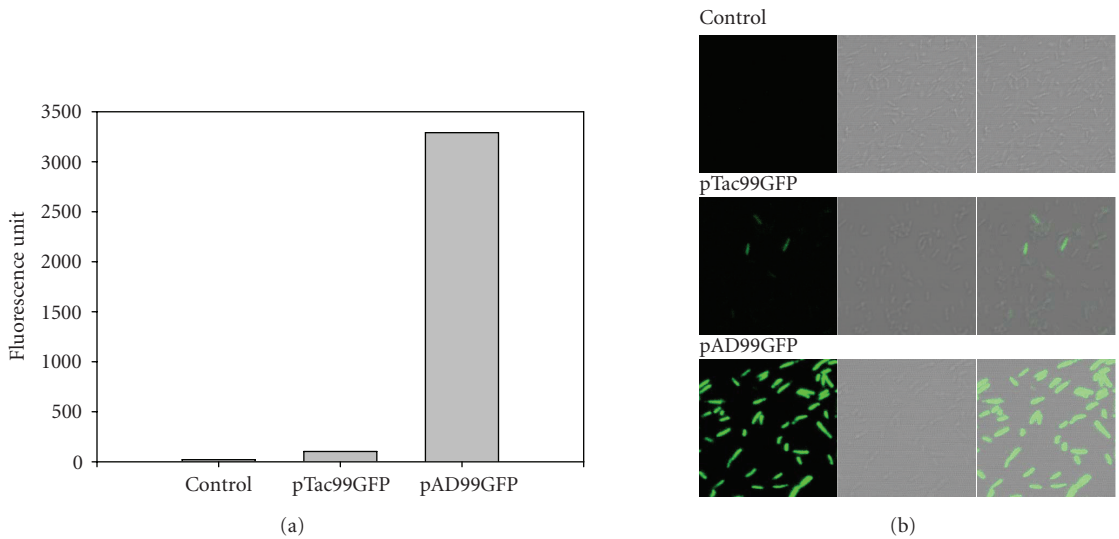

Figure 6: The fluorescence intensities (a) and confocal microscopic images (b) of *E. coli* W3110 cells by induction with IPTG (middle panels) or oleic acid (bottom panels). As a control, the *E. coli* strain without plasmid is also shown (top panels). Shown in (b) are immunofluorescence micrographs (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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