RESEARCH ARTICLE

Expression profiling of proteins in L-threonine biosynthetic pathway of *Escherichia coli* by using antibody microarray

Min-Kyu Han[1], Mi-Young Hong[1], Dohoon Lee[1], Dong-Eun Lee[1], Geon Youp Noh[2], Jin-Ho Lee[3], Sung-Ho Kim[2] and Hak-Sung Kim[1]

[1] Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Guseong-dong, Daejeon, Korea
[2] Division of Applied Mathematics, Korea Advanced Institute of Science and Technology, Guseong-dong, Daejeon, Korea
[3] R&D Center of Bioproducts, Institute of Science and Technology, CJ Corp., Gyeonggi-do, Korea

We demonstrate the use of an antibody (Ab) microarray for a comparative expression profiling of proteins in an L-threonine biosynthetic pathway of *Escherichia coli* between a parental strain (W3110) and L-threonine overproducing mutant (TF5015). On the basis of a global comparative transcriptome analysis between the two strains, 28 analytical target proteins were selected and subjected to a production of polyclonal Abs against them. An Ab microarray was constructed by spotting a set of produced antibodies on a glass slide, and was employed for a comparative expression profiling of the proteins between the two strains by a two-color fluorescence assay method. The performance of the Ab microarray was evaluated with respect to cross-reactivity of the antibodies, dye-labeling efficiency, and the nature of antigenic proteins. Of these, the cross-reactivity of the used antibodies was found to mainly cause the deviation of the observed expression ratios from the expected ones. To offset the deviations, correction factors were derived from a statistical analysis and introduced. As a result, ten proteins were categorized to be up-regulated, while one was down-regulated in TF5015. Expression profiling of proteins using the Ab microarray was further verified by comparison with Western blotting and 2-DE.

Received: May 1, 2006
Revised: July 14, 2006
Accepted: July 17, 2006

Keywords:
Antibody microarrays / Cross-reactivity / Expression profiling / L-threonine biosynthesis

Correspondence: Professor Hak-Sung Kim, Department of Biological Sciences, Korea Advanced Institute of Science and Technology 373-1, Guseong-dong, Yuseong-gu, Daejeon 305-701, Korea
E-mail: hskim76@kaist.ac.kr
Fax: +82-42-869-2610

Abbreviations: Ab, antibody; **cat**, chloramphenicol acetyltransferase; **His_6**, hexahistidine; **IgG**, immunoglobulin G; **KO**, knockout; **LB**, Luria-Bertani; **MBP**, maltose binding protein; **NHS**, *N*-hydroxysuccinimide

1 Introduction

In the post-genomic era, the wealth of proteomic information and complexity of intracellular signaling processes have required an efficient analytical approach to accomplish a rapid, low-volume and multiplexed assay in a high-throughput manner [1, 2]. Such methods can be effectively used for basic biological research, diagnoses of diseases, identification of therapeutic markers and targets, and profiling of responses to toxins and pharmaceuticals [3–9]. A large num-

ber of studies have shown the utility of antibody (Ab) arrays for the quantitation of specific proteins and the discovery of biomarkers based on a comparative profiling of the protein expression between sample and reference pools [7, 10, 11]. In addition, the feasibility and performance of Ab microarrays were evaluated with respect to parallel quantitation for antigen-antibody interactions and parameters for microarray fabrication [12–15]. Notable advances have been made in the development of an Ab microarray, offering some advantages in terms of sample volume, high-throughput analysis, and simplicity over conventional methods, including Western blotting and 2-DE. However, despite a number of studies regarding the utility of an Ab microarray, its reliability in the expression profiling of proteins remains a challenge [16].

Here, we demonstrate the use of an Ab microarray for a comparative expression profiling of proteins in the L-threonine biosynthetic pathway of *Escherichia coli* between W3110 and TF5015 through a two-color fluorescence assay. For this, 28 target proteins involved in L-threonine biosynthesis were selected on the basis of our previous study on a transcriptome analysis between the two strains [17], and were then subjected to a production of polyclonal Abs. We constructed an Ab microarray with the produced antibodies, and investigated the factors affecting the performance of the Ab microarray. Expression profiling of the proteins between the two strains was performed with reasonable reliability through a statistical analysis. The utility of the microarray was further verified by comparison with Western blotting and 2-DE, the details of which are reported here.

2 Materials and methods

2.1 Expression and purification of *E. coli* proteins

For the amplification of target genes as listed in Table 1, a set of primers for each gene was designed based on a respective sequence taken from a Swiss-Prot database. Each gene was PCR-amplified from the genomic DNA of an *E. coli* W3110 strain using *Pfu* DNA polymerase and corresponding primers. Genes coding ThrA, PtsG, and OppA were cloned into a constitutive expression vector system, pHCEIA (NcoI) (Takara, Shiga, Japan), with the insertion of a hexahistidine (His_6)-tag at the N terminus. In the case of PtsG, only the cytoplasmic domain [18] was cloned because serious cell lysis occurred when the whole protein was overexpressed. The genes coding for Ppc, AceB, ThrC, and a C-terminal fragment of Ppc monomer were cloned into pMAL-c2x (New England Biolabs, Beverly, MA, USA) for a maltose binding protein (MBP)-fused expression. All remaining genes were cloned into pQE30 (Qiagen, Chatsworth, CA, USA) for a His_6-tag fusion at the N terminus. All restriction reactions of plasmid were treated with calf intestinal alkaline phosphatase to prevent self ligation. The constructed plasmid was transformed into an *E. coli* JM109 by electroporation, and transformed cells were cultured in a Luria-Bertani (LB) medium. When OD_{600} nm reached about 0.5, *E. coli* cells harboring inducible vectors (*e.g.*, pQE30 and pMAL-c2x) were induced by addition of isopropyl-β-D-thiogalactopyranoside into a culture medium at a final concentration of 1 mM. As for pHCEIA (NcoI), host cells were cultured for 12 h to maximize the expression level of protein.

His_6-tagged and MBP-fused proteins were purified from *E. coli* lysates according to the manufacturer's instructions. His_6-tagged proteins were loaded to Ni-nitrilotriacetic acid resin followed by elution with a 250 mM imidazole solution. MBP-fused proteins purified through amylose resin were treated with Factor Xa and further purified according to the manufacturer's instructions. Purity of the acquired proteins was confirmed by SDS-PAGE (Fig. 1A, top). Protein concentration was determined using a standard protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples were stored at −20°C until further use.

2.2 Construction of *E. coli* knockout mutant

A specific gene of *E. coli* was deleted according to the method by Datsenko *et al.* with a slight modification [19]. Briefly, 500 nucleotides at the N and C termini of the target gene were PCR-amplified from the genomic DNA of W3110. In parallel, the chloramphenicol acetyltransferase (cat) gene was amplified from pKD3. The acquired three PCR products were assembled by overlapping PCR in the order of N-terminal, cat, and C-terminal sequences. The integrated sequences were transformed into pKD46-harboring competent cells induced by arabinose to express a λ Red recombinase system. The transformed cells were then incubated for 8 h at room temperature to give rise to recombination between transformed and genomic DNA sequences. Replacement of the target gene in the host cells with transformed DNA renders cells resistant to chloramphenicol, and antibiotic-resistant transformants can be selected on a LB-agar medium containing 25 μg/mL chloramphenicol. Construction of a knockout (KO) mutant was finally verified by PCR.

2.3 Preparation of Abs

Abs against 28 target proteins of *E. coli* were produced from a rabbit according to standard procedures [20]. Briefly, about 200 μg purified antigenic protein was mixed with Freund's complete adjuvant, followed by the first injection into the rabbit. Every 2 weeks, the same amount of antigen containing Freund's incomplete adjuvant was introduced into the same rabbit until the induction of each Ab was detected. The generation of the respective Ab in serum was confirmed by Western blotting. Each Ab against the cognate antigen was purified from the serum for construction of the Ab microarray. Briefly, cyanogen bromide-activated Sepharose resin (Sigma-Aldrich, St. Louis, MO, USA) was first activated by rinsing with refrigerated 1 mM HCl and a conjugation buffer (0.1 M sodium bicarbonate buffer containing 0.25 M NaCl, pH 8.3). The activated resin was incubated

Table 1. List of *E. coli* proteins used in Ab microarray assays

ID no.	Gene name	Protein name	Function	Correction factor[a)]
0		Anti-human immunoglobulin G antibody	Internal standard for normalization	–
1	ppc	Phosphoenolpyruvate carboxylase	Energy metabolism	2.083
2	thrA	Aspartokinase/homoserine dehydrogenase I	Amino acid biosynthesis	2.582
3	gapA	Glyceraldehyde 3-phosphate dehydrogenase A	Energy metabolism	2.537
4	pfkA	6-Phosphofructokinase isozyme 1	Energy metabolism	2.386
5	ptsG	PTS system, glucose-specific IIBC component	Transport	2.437
6	aceA	Isocitrate lyase	Cell Structure	2.270
7	gltA	Citrate synthase	Central intermediary metabolism	2.347
8	proA	Gamma-glutamyl phosphate reductase	Amino acid biosynthesis	2.084
9	pgk	Phosphoglycerate kinase	Energy metabolism	2.261
10	fba	Fructose-bisphosphate aldolase class II	Energy metabolism	1.716
11	sdhA	Succinate dehydrogenase flavoprotein subunit	Energy metabolism	2.252
12	pgi	Glucose-6-phosphate isomerase	Energy metabolism	2.361
13	pgm	Phosphoglucomutase	Carbon compound catabolism	2.325
14	pckA	Phosphoenolpyruvate carboxykinase	Central intermediary metabolism	1.604
15	mdh	Malate dehydrogenase	Energy metabolism	1.520
16	pfkB	6-Phosphofructokinase isozyme 2	Energy metabolism	2.482
17	dapB	Dihydrodipicolinate reductase	Amino acid biosynthesis	2.174
18	zwf	Glucose-6-phosphate 1-dehydrogenase	Energy metabolism	2.172
19	eno	Enolase	Energy metabolism	2.435
20	icdA	Isocitrate dehydrogenase	Central intermediary metabolism	1.776
21	aspC	Aspartate aminotransferase	Amino acid biosynthesis	1.451
22	asd	Aspartate-semialdehyde dehydrogenase	Amino acid biosynthesis	1.819
23	thrB	Homoserine kinase	Amino acid biosynthesis	2.034
24	aceB	Malate synthase A	Cell structure	2.158
25	thrC	Threonine synthase	Amino acid biosynthesis	2.053
26	yfiD	Hypothetical protein yfiD	Energy metabolism	1.922
27	ahpC	Alkyl hydroperoxide reductase subunit C	Sulfate starvation-induced protein	1.547
28	oppA	Periplasmic oligopeptide-binding protein	Transport	2.101
29		Anti-bovine serum albumin antibody	Negative control	–

a) Correction factors were estimated through statistical analysis of the data from the calibration tests as described in the Materials and Methods section.

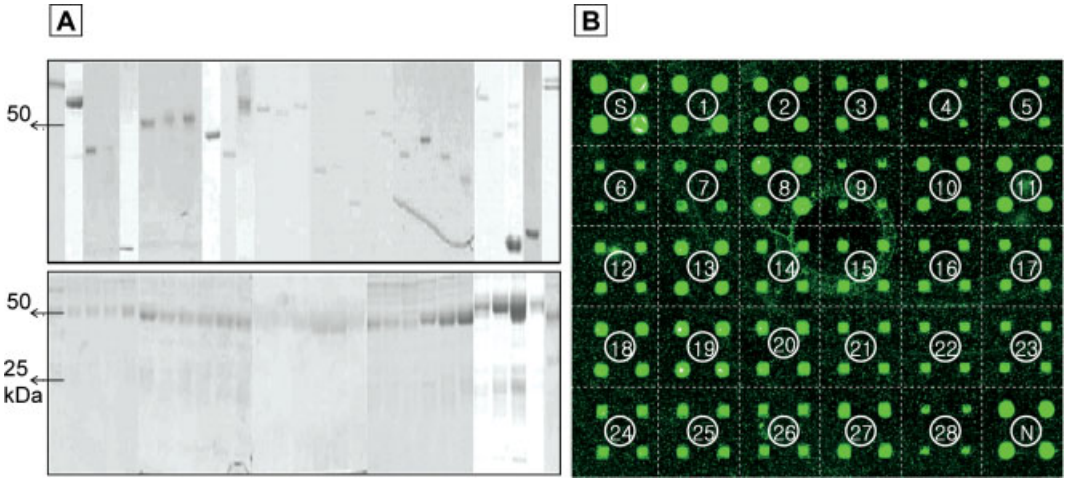

Figure 1. (A) SDS-PAGE of purified target proteins from *E. coli* (top) and affinity-purified antibodies against cognate proteins (bottom). (B) Layout of Ab spots in a microarray for profiling of differential expression of 28 target proteins, including an internal standard (S) and a negative control (N). Spotted antibodies in quadruplicate were visualized using Cy3-labeled protein A.

overnight with an antigenic protein solution (~8 μM) at 4°C. After the remaining reactive groups of the antigen-coupled resin were blocked by immersion in a 1 M Tris-HCl buffer (pH 8.0) at room temperature for 2 h, the blocked resin was packed in a column under atmospheric pressure. To remove simply adsorbed proteins, the antigen-coupled resin was then washed by flowing ten volumes of the 0.1 M sodium acetate buffer containing 0.5 M NaCl (pH 4.0) and PBS. After a thorough washing with PBS, the column was incubated with diluted anti-serum for 3 h at room temperature, followed by a washing with ten volumes of PBS. Ab bound to the antigen-coupled resin was finally eluted by running 0.2 M glycine-HCl (pH 2.7). Eluted Ab fractions were immediately neutralized, collected, and desalted with PBS. All purified Abs were concentrated to about 0.5 mg/mL with an Amicon concentrator (Millipore, Billerica, MA, US), analyzed to estimate the purity using SDS-PAGE (Fig. 1A, bottom), and then stored below −20°C until further use.

2.4 Preparation and dye-labeling of proteins

For cultivation of both strains, a 250-mL flask was filled with 20 mL fermentation medium (pH 7.0) containing 1.4 g glucose, 40 mg KH_2PO_4, 340 mg $(NH_4)_2SO_4$, 20 mg $MgSO_4 \cdot 7H_20$, 0.2 mg $MnSO_4 \cdot 4H_2O$, 0.2 mg $FeSO_4 \cdot 7H_2O$, 0.6 g $CaCO_3$, 3 mg methionine, and 40 mg yeast extract [17]. When growth of the cells in the flask reached an early stationary phase, a aliquot of 1.5 mL was centrifuged, re-suspended with PBS, and then lysed using a sonicator (Sonics & Material, Newtown, CT, USA). Following the separation of a supernatant by centrifugation, the resulting *E. coli* lysates were dialyzed using a membrane with a molecular cutoff of 12 kDa (Spectrum medical industries, LA, CA, USA) to remove free amino acids in lysates for an efficient labeling of fluorescent dyes. As the internal standard protein for normalization of detected signals, human immunoglobulin G (IgG) was added to the dialyzed lysates, and final concentrations of IgG and lysate were adjusted to be 200 ng/mL and 1 mg/mL, respectively.

To probe the differential expression of target proteins between W3110 and TF5015 on the Ab microarray, cell lysates and other proteins were labeled with *N*-hydroxysuccinimide ester (NHS)-linked Cy3 or Cy5 dye (Amersham Biosciences, Buckinghamshire, UK). Either Cy3 or Cy5 dye was added into the cell lysate (1 mg/mL in PBS) in the dark, and the reaction mixture was placed for 30 min at room temperature with a gentle mixing every 10 min. After the coupling reaction was stopped by the addition of 1 M Tris-HCl (pH 8.0), dye-labeled proteins were separated from free dyes using gel filtration chromatography and concentrated to 1 mg/mL. From spectroscopic titration using a UV-Vis spectrophotometer, a labeled ratio of dye to protein was found to be about 1 for the lysates from both strains. To label and inspect the integrity of the Ab spots, a mixture of protein A from *Staphylococcus aureus* (10 μg/mL) and ovalbumin (1 mg/mL) was labeled with NHS-linked Cy3 dye and used. For assessment of the cross-reactivity of antibodies, a mixture of purified proteins (concentration of each protein was 200 ng/mL) containing ovalbumin (1 mg/mL) was labeled with either Cy3 or Cy5 dye and used.

2.5 Fabrication of Ab microarray and read out

The Ab microarray was constructed by spotting antibodies onto an epoxy-coated glass slide (Slide E; Schott Nexterion, Jena, Germany) using a robotic arrayer (Microsys, Cartesian Technologies, Irvine, CA, USA) equipped with CMP 3 spotting pins (Telechem International, Sunnyvale, CA, USA). As shown in Fig. 1B, a single Ab microarray was composed of a set of 28 antibodies raised against *E. coli* proteins, anti-human IgG (Sigma-Aldrich, St. Louis, MO, USA), and anti-BSA (Sigma-Aldrich) as the internal standard and negative control, respectively, and each Ab was spotted in quadruplicate. All protein solutions for spotting were prepared in PBS supplemented with 0.5% trehalose, and a relative humidity of the arrayer was maintained at 70%. Following incubation at room temperature for 3 h after spotting, the Ab-spotted slide was taken out of the arrayer and subsequently blocked with PBS supplemented with either 5% BSA, 3% TopBlock (Fluka, Buchs, Switzerland), or 5% skimmed milk for 3 h. After a thorough washing with PBS, the Ab microarray was incubated with dye-labeled biological samples in the dark for 1 h. Prior to fluorescence detection, the Ab microarray was consecutively washed with PBS, PBS containing 0.1% Tween-20 (PBST), and distilled water followed by drying with N_2 gas. Fluorescence intensities emitted from Cy3- and Cy5-specific channels were read out using a fluorescence scanner (GenePix Personal 4100A; Axon instruments, Union City, CA, USA), and analyzed using GenePix Pro 4.1 software provided by the manufacturer. Intensities of Cy3 and Cy5 taken from all spots were corrected for background intensity, subjected to normalization against the average intensity of Cy5/Cy3 estimated from anti-human IgG spots, and their median values were collected. Raw spots showing either obvious defects or an S/N less than 2 were discarded prior to further analysis. In this way, assays of the Ab microarray were carried out in triplicate each day for 3 days, and accordingly, the resulting ratios represent the average of 32 values for each Ab spot.

To assess the extent of cross-reactivity of Ab, the lysates of W3110 and its KO mutants were used. The lysates from KO mutants and W3110 were separately labeled with Cy5 dye as described above, and each lysate was loaded onto the Ab microarray. If an Ab has no cross-reactivity, no fluorescence intensity would be observed from the Ab when lysate from a KO mutant is applied to the Ab microarray because the Ab-specific antigenic protein is not expressed. On the contrary, an Ab with high cross-reactivity would display high Cy5 intensity even for lysate from a KO mutant. Thus, it is inferred that the relative fluorescence intensity from lysate of a KO mutant against that from W3110 on the respective Ab spot can represent the degree of cross-reactivity. In this

regard, we compared the signal intensity of Cy5-labeled KO lysate with that of Cy5-labeled W3110 lysate for specific antibodies after normalization of array-to-array variation using an internal standard. A set of these data *versus* the measured ones from Fig. 2A were then subjected to K-means analysis to evaluate the extent of cross-reactivity of each Ab and deviation of the measured ratio from the expected one.

2.6 Statistical analysis

The measured ratios using a comparative fluorescence assay were found to be biased from the expected ones, and a correction factor was introduced to offset the deviations. To this end, we performed calibration tests using a set of artificially mingled *E. coli* lysates. Experimentally obtained data were subjected to regression analysis where a measured ratio from the target protein was defined as a predictor variable (X_{ij}, $i = 1, 2, \ldots, 9$, $j = 1, 2, \ldots, n_i$) and an expected, real one as a response variable (Y_{ij}, $i = 1, 2, \ldots, 9$, $j = 1, 2, \ldots, n_i$). Accordingly, a no-intercept regression model was assumed [$y_{ij} = \hat{\beta}\, x_{ij} + \varepsilon_{ij}$, $\varepsilon_{ij} \sim N(0, \sigma^2)$], and this seemed appropriate to an analysis of the Ab microarray data. In this model, the value of $\hat{\beta}$ was estimated for each Ab by a least square method ($y_{ij} = \hat{\beta}\, x_{ij}$), and correspondingly, appropriateness of the model was assessed based on the coefficient of determination (R^2). On the other hand, a K-means analysis was employed to demonstrate and classify the seriousness of cross-reactivity of the Ab for analysis of *E. coli* lysates. In the process of data-mining clustering, we first partitioned experimentally obtained items into K initial clusters, processed through the list of items, and then assigned an item to the cluster whose centroid (mean value) is nearest. In a serial process, the second step was repeated until no more reassignment took place, as the centroid was recalculated for the cluster receiving a new item and for the cluster losing the item. Meanwhile, the effect of alternative labeling of Cy5 and Cy3 dyes was tested for W3110 and TF5015 lysates. The outcome was represented by estimation of a Cy5/Cy3 intensity ratio for a given condition, multiplication by a measured ratio from a reverse labeling test, and then conversion into a $\log_2$ base. If the ultimate value is close to 'zero', this denotes no effect of alternative labeling on dye-labeling efficiency for the corresponding Ab.

2.7 Western blotting, 2-DE, and MALDI-MS

Cell lysate (100 μg) from either W3110 or TF5015 was prepared as described above and applied to SDS-PAGE for separation. The developed gel was equilibrated with transfer buffer (30 mM glycine, 48 mM Tris, 0.037% SDS, and 20% methanol, pH 8.3), and proteins on the gel were electrotransferred onto an NC membrane (Amersham Biosciences, Buckinghamshire, UK). The blotted membrane was incubated with a 1000-fold diluted antiserum raised against each protein, and then with a 2000-fold diluted anti-rabbit IgG conjugated with a horseradish peroxidase. Ab-bound proteins were visualized using an immuno-blot assay kit (Bio-Rad), and the thicknesses of the visible bands were estimated using ImageJ software (National Institute of Health, Bethesda, MD, USA).

2-DE and MALDI-TOF MS were carried out according to procedures described previously [17]. Briefly, W3110 and TF5015 lysates were dissolved in 400 μL rehydration buffer (8 M urea, 2% CHAPS, 18 mM DTT, 0.5% IPG buffer) and loaded onto 18-cm IPG strips (pH 3–10 L type) (Amersham Biosciences). Following incubation for 12 h, proteins were focused on the IPG strip by applying a serial of voltages at 500 V for 1 h, 1000 V for 1 h, and finally, 8000 V for 12 h. Thereafter, separation of proteins in the second dimension was conducted by SDS-PAGE. After fixation and CBB staining, a 2-DE gel was scanned, and densities of spots were averaged from three different gel images. Protein spots displaying distinctly different intensities between W3110 and TF5015 were excised followed by digestion with a 20 ng sequencing-grade trypsin (Promega, Madison, WI, USA). A pool of digested peptides was analyzed by MALDI-MS (Voyager-DE STR, Applied Biosystems, Drive Foster City, CA, USA). In a peptide fingerprint, peaks derived from

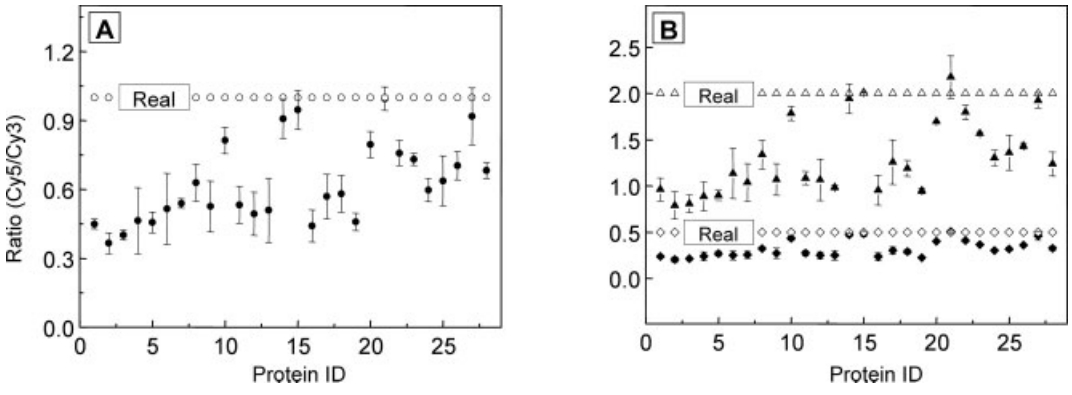

Figure 2. Measured ratios (closed symbol) and real ratios (open symbol) for target proteins when mixtures of Cy5- and Cy3-labeled W3110 lysates with different ratios (Cy5/Cy3) were applied to the Ab microarray: (A) Cy5/Cy3 = 1.0, (B) 0.5 and 2.0, respectively.

autodigestion of trypsin were used as an internal calibration. Trypsin-digested protein was identified using Protein Prospector (http://prospector.ucsf.edu/) and based on comparison with an *E. coli* reference gel provided by SWISS-2DPAGE (http://tw.expasy.org/ch2d//).

3 Results and discussion

3.1 Construction of Ab microarray

We previously conducted comparative analyses of transcriptome and proteome between prototropic *E. coli* W3110 and L-threonine overproducing TF5015 strains to get some insight into global regulation in modulating an L-threonine biosynthetic pathway using a DNA macroarray and 2-DE integrated with MALDI-MS [17]. As a result, 54 of 4290 genes in total (1.3%) were found to give rise to differential expression profiles in TF5015. The current analytical methods are unlikely to precisely decipher the differential expression of all kinds of proteins in biological systems. The 2-DE technique is known to be ineffective for the analysis of low-abundance, large, and hydrophobic proteins such as membrane proteins [21]. In contrast, Ab microarrays have been reported to capture and detect even large or low-abundance proteins in complex biological samples with sufficient sensitivity in a high-throughput manner [6, 13]. In this context, we attempted a microarray-based approach to identify differentially expressed proteins in an L-threonine biosynthesis pathway between W3110 and TF5015. For this, we selected 28 target genes and corresponding proteins, which appear to be linked with the L-threonine over-production ability of TF5015, based on a transcriptome analysis [17]. As summarized in Table 1, most of the selected proteins are involved in energy metabolism, amino acid biosynthesis, catabolism of the carbon compound, central intermediary metabolism, cell structure, sulfate starvation response, and transport [17].

Antibodies against the 28 selected target proteins were obtained from sera of immunized rabbits through affinity purification using corresponding target proteins (Fig. 1A). A set of 28 antibodies, together with antibodies as an internal standard (anti-human IgG) for normalization and a negative control (anti-BSA), were arrayed onto an epoxy-coated glass slide in quadruplicate for each Ab. The layout of the spotted antibodies was then probed by incubating the prepared Ab microarray with a solution of Cy3-labeled protein A (Fig. 1B). Spots with uniform morphology and strong fluorescence emission indicate reliability of the arraying conditions used in this work.

3.2 Ab microarray assays

The constructed Ab microarray was subjected to analysis of complex biological samples. When the Ab microarray was treated with *E. coli* lysates, intensities of background signals were usually less than 5% of the genuine signal intensities from Ab spots. This indicates that use of 5% BSA, 3% Top-Block, or 5% skimmed milk was effective for the blocking of non-spotted areas, preventing nonspecific adsorption of proteins. Signals from Ab spots were corrected against the background signal and normalized using the average value of the Cy5/Cy3 intensity ratio from internal standard spots. In a two-color fluorescence assay, a number of normalization methods have been attempted to compensate for the discrepancies in labeling efficiencies and quantum yields of dyes [22]. In this study, human IgG as an internal standard was added in the same quantity into two different biological samples for labeling with Cy3 and Cy5 dyes.

We first tested whether the constructed Ab microarray yields a reliable profiling of protein expressions. Several reports have shown that the experimentally observed ratios using protein microarrays often display serious deviation from the expected, real ones in comparative analysis [2, 5, 12]. For instance, Miller *et al.* [5] reported that Ab microarray assays and ELISA revealed significant variation in correlation parameters when the abundance of proteins between cancer and normal sera were analyzed. In this context, we conducted calibration tests using a set of *E. coli* lysates with a predetermined ratio of dye-labeled proteins to examine whether the measured ratios from Ab microarray assays are well correlated with the expected ones. Cy5- and Cy3-labeled W3110 lysates were mixed at three different ratios, 0.5-, 1.0-, and 2.0-fold (Cy5/Cy3), and applied to Ab microarrays. As a result, we observed the deviation of the measured ratios from the expected ones for all the tested ratios (Fig. 2). This discrepancy seemed to be caused by various factors including cross-reactivity of Ab, inconsistency of dye-labeling efficiency, low concentration of antigens, and loss of Ab reactivity during spotting [5]. It is inferred that direct use of the experimentally measured ratios does not reflect a real one in a biological sample, leading to erroneous interpretation of the expression profiling of proteins.

3.3 Factors affecting the performance of Ab microarray

To gain some insights into the factors causing the discrepancy between the measured ratios and the expected ones, we first assessed the cross-reactivities of spotted antibodies. The lysates from W3110 and its KO mutants lacking specific genes were used. Seven proteins were chosen by taking into consideration the extent of deviation from the real ratios (Fig. 2). The lysates of KO mutants and W3110 were separately labeled with Cy5 dye, and each lysate was applied to an Ab microarray. We reasoned that comparative analysis of the fluorescence intensity of a given spot between KO mutant and W3110 lysates would reveal the extent of cross-reactivity of the corresponding Ab. We determined the relative intensities from seven Ab spots between the Cy5-labeled KO mutant and Cy5-labeled W3110 lysates, and plotted them against the observed ratios of selected proteins that

were obtained from the calibration test shown in Fig. 2A for K-means clustering analysis. As a result, the antibodies against the selected proteins were classified into two groups, depending on the extent of deviation as shown in Fig. 3A. Antibodies against Mdh (Table 1; no. 15) and AhpC (no. 27) gave rise to little discrepancy between the observed ratios and the expected ones, implying negligible cross-reactivities. The cross-reactivity of anti-PckA (no. 14) was found to be moderate. In contrast, four other proteins showed a large deviation of the measured ratios from the expected ones, indicating serious cross-reactivities. These results were well coincident with those obtained from the calibration tests of the Ab microarray (Fig. 2). Taken together, it is likely that the cross-reactivity of used antibodies had a significant effect on the discrepancy between the measured ratios and the expected ones. Consequently, the deviation of the measured ratio from the expected one became larger as the cross-reactivity of the given Ab was higher.

To further confirm the effect of cross-reactivity of the Ab, we carried out supplementary tests using purified proteins. Six proteins used in the KO mutant experiment and Pgk (no. 9) were purified, labeled with either Cy5 or Cy3, and a 1.0-fold (Cy5/Cy3) mixture of the dye-labeled proteins was applied to the Ab microarray. As shown in Fig. 3B, the purified proteins resulted in a smaller deviation of the measured ratios from the expected ones (in this case, 1.0) compared to the W3110 lysate, except for Ppc (Table 1; no. 1, see below). Similar results were obtained for the mixtures with different dye-labeled protein ratios (Cy5/Cy3 = 0.5 and 2.0) (data not shown). Furthermore, a mixture of the six purified proteins, which have a negligible cross-reactivity for non-cognate antibodies, showed a similar trend (Fig. 3C). Unlike the *E. coli* lysate, a purified protein or a mixture of purified proteins gave rise to little deviation of the measured ration from the expected ones. Based on these results, it is plausible that cross-reactivity of Ab for other irrelevant proteins in lysates

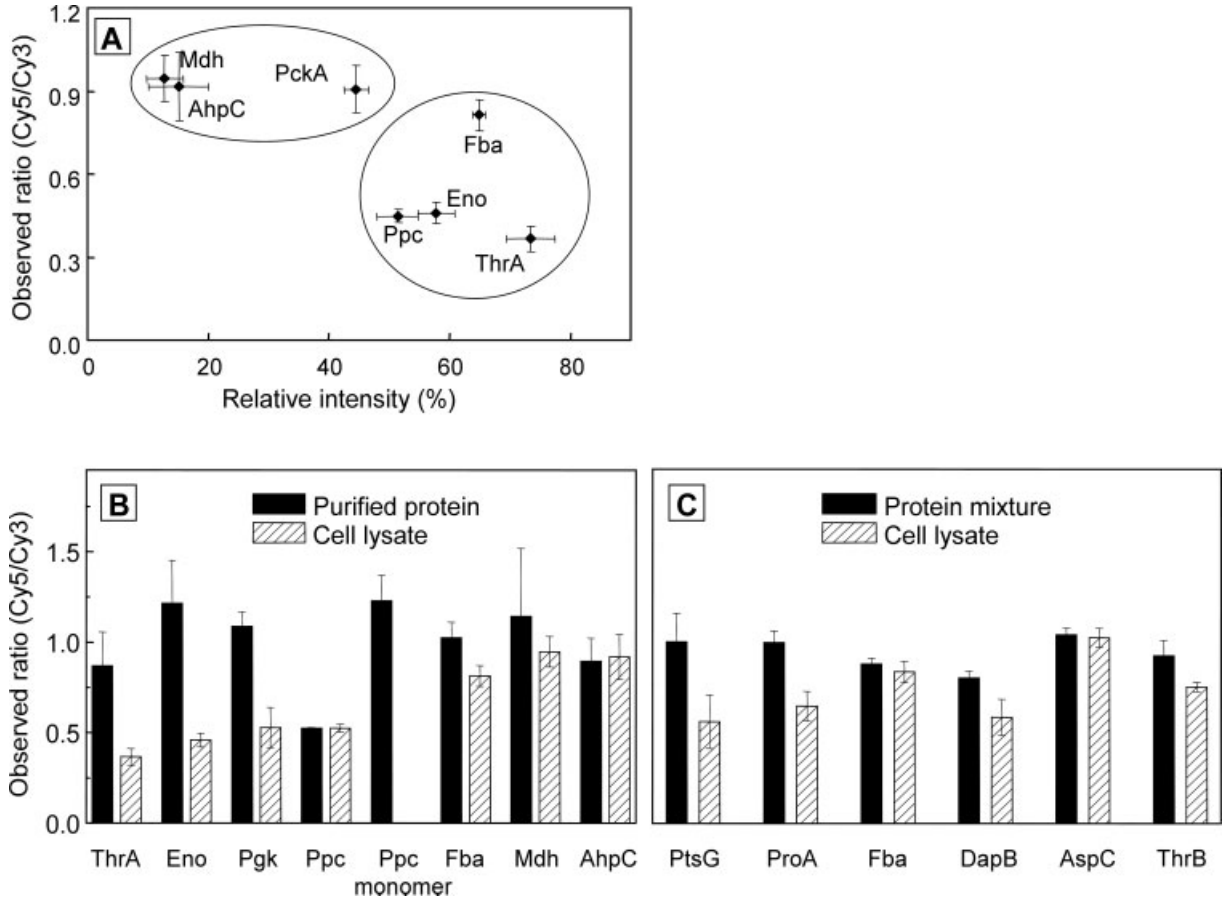

Figure 3. (A) Classification of the tested antibodies, depending on the extent of cross-reactivity. Relative intensity (%) indicates the intensity of Cy5-labeled KO lysate over Cy5-labeled W3110 lysate. The y-axis represents the observed ratio (Cy5/Cy3) when a mixture of the same quantity of Cy5- and Cy3-labeled W3110 lysates was applied to the Ab microarray for calibration test as shown in Fig. 2A. Data represent the average values and SDs in triplicate experiments. (B) Comparison of observed ratios (Cy5/Cy3) for purified target proteins and W3110 lysate. Ppc monomer indicates the monomeric subunit of Ppc. (C) Comparison of observed ratios (Cy5/Cy3) for a mixture of other purified proteins. Equal amounts of Cy5- and Cy3-labeled proteins were mixed and applied.

mainly caused the serious deviation of the observed ratios from the expected ones.

As shown in Fig. 3B, Ppc resulted in a large discrepancy between the observed ratio and the expected one. Unlike other tested proteins, this deviation was not mitigated even when a purified form of Ppc was used. Thus, we reasoned that the size of the protein might affect the result, because Ppc is a homotetrameric protein and has a relatively large molecular mass (396 kDa). The size effect was investigated using a C-terminal fragment of Ppc monomer (27 kDa) instead of an intact multimeric large protein. The use of the purified monomeric Ppc yielded a measured ratio approaching the real one (Fig. 3B), which indicates that the size of target proteins has an effect on the performance of the Ab microarray.

The performance of the Ab microarray might also be affected by production parameters such as dye-labeling efficiency, concentration of antigenic protein, surface chemistry, and nonspecific adsorption of protein. Haab *et al.* [12] reported that the inconsistency in dye-labeling efficiency and the concentration of antigenic protein can cause incorrect results in protein microarray analysis. To examine the effect of labeling efficiency, alternative labeling of W3110 and TF5015 lysates were carried out. The obtained ratios (Cy5-W3110/Cy3-TF5015) were multiplied by the ratios (Cy5-TF5015/Cy3-W3110) acquired after a reverse labeling of W3110 and TF5015 lysates. The calculated value was then transformed in a $\log_2$ base. As can be seen in Fig. 4, the measured values from more than 20 spots approached zero (= $\log_2 1$), indicating little difference in labeling efficiency between the two dyes. Exceptionally, spots of six antibodies raised against GapA (Table 1; no. 3), PfkA (no. 4), SdhA (no. 11), Pgi (no. 12), Pgm (no. 13) and PfkB (no. 16) displayed somewhat lower values than zero. This result seemed to be caused by a difference in dye-labeling efficiency or a mal-function of spotted antibodies due to loss of inherent conformation of the Ab molecules. In our experiments, the ratios of antigenic proteins varied in a narrow range from 0.5 to 2.0, but these ratios changed three orders of magnitude from 0.01 to 10 in the study of Haab *et al.* [12]. In addition, the concentration of antibodies used in our work was adjusted to be about 0.5 mg/mL, and the total protein concentration of sample solution was fixed at about 1 mg/mL. These values are within the concentration ranges that are generally accepted as optimal for reliable protein microarray analysis [15]. Thus, the concentrations of Ab and analyte proteins are unlikely to have negative effects on the performance of the Ab microarray. Collectively, expression profiling of proteins using the Ab microarray was influenced by inherent factors including the size of the protein, dye-labeling efficiency, and specificity of antibodies. Of them, the cross-reactivity of antibodies seemed to have the most serious effect on the performance of the Ab microarray.

3.4 Profiling of protein expression

In an attempt to offset the deviation of the measured ratios from the expected ones, correction factors were introduced in the analysis of Ab microarray data. In definition, the magnitude of a correction factor reflects the extent of deviation of an observed ratio from the expected one. Correction factors were determined through statistical analysis of the calibration data as a function of the expected ones as described in the Materials and Methods section. The calculated correction factors for corresponding antibodies are listed in Table 1. The measured ratios from Ab microarray were multiplied by correction factors for corresponding antibodies and used for profiling of a protein expression.

The developed Ab microarray was treated with a mixture of Cy3-labeled W3110 and Cy5-labeled TF5015 lysates, and then the obtained signals were normalized using a Cy5/Cy3 intensity ratio of anti-human IgG spots. As shown in Fig. 5A, all spotted antibodies except anti-BSA showed strong fluorescence intensities, and their S/N were found to be higher than 2 except anti-BSA, implying that the Ab microarray worked well. Signals at positions numbers 3 and 26 appear to display a green color by the naked eye. This seems to be due to the fact that the S/N of the Cy3 signal at these Ab spots was

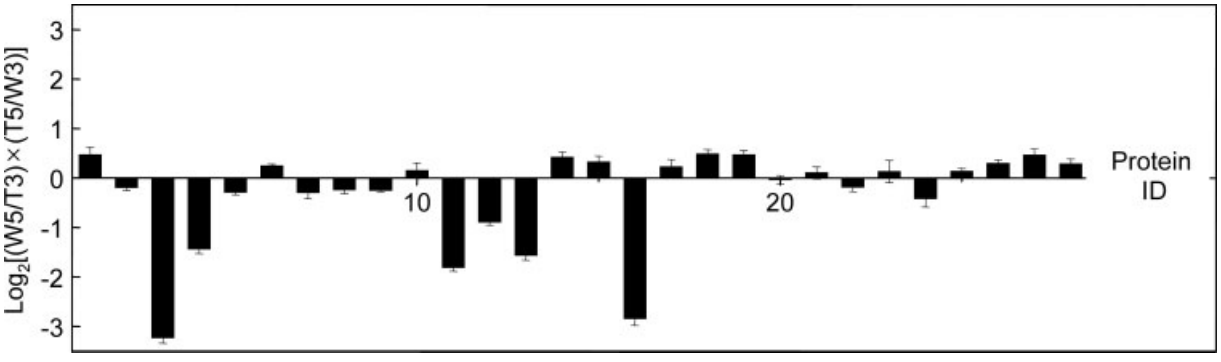

Figure 4. Effect of alternative dye labeling. Logarithmic values of Cy5-labeled W3110 (W5) over Cy3-labeled TF5015 (T3) multiplied by Cy5-labeled TF5015 (T5) over Cy3-labeled W3110 (W3) for 28 antibodies were determined. Data represent the average values and SDs in quadruplicate experiments.

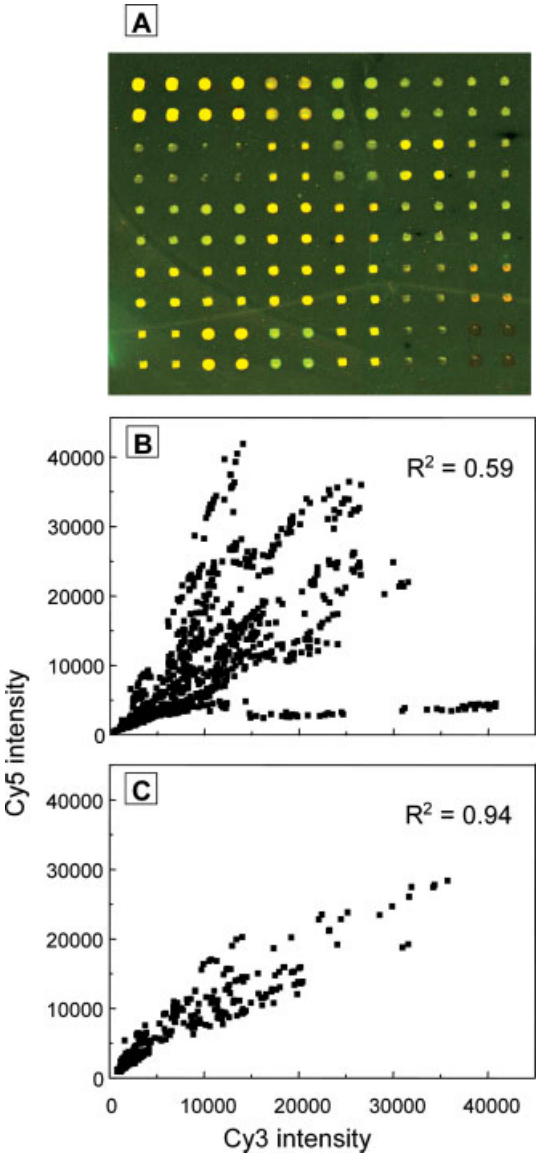

Figure 5. (A) Representative fluorescence images of the Ab microarray after incubation with a mixture of Cy5-labeled TF5015 and Cy3-labeled W3110 lysates. Scattered plots of Cy5 *versus* Cy3 intensities taken from an Ab microarray after treatment with a binary mixture of Cy3-labeled W3110 and Cy5-labeled TF5015 (B) and Cy3- and Cy5-labeled W3110 lysates (C). In all tests, two differently labeled *E. coli* lysates were mixed in equal quantity.

much higher than that of Cy5. However, Cy5-labeled TF5015 lysate evidently expressed the corresponding proteins when analyzed using a scanner. We compared the intensity distributions of the mixture of Cy3- and Cy5-labeled W3110 lysates with that of Cy3-labeled W3110 and Cy5-labeled TF5015 lysates, and observed that the latter resulted in a more widely scattered distribution (R^2 = 0.59) of Cy5 *versus* Cy3 intensities (Fig. 5B) than the former (R^2 = 0.94) (Fig. 5C). This result indicates that the developed Ab microarray can decipher the difference in protein expression level between W3110 and TF5015.

We performed the protein expression profiling between Cy3-labeled W3110 and Cy5-labeled TF5015 lysates by using the developed Ab microarray. Thirty two values of Cy5/Cy3 ratio were acquired from 8 independent experiments for each target protein, normalized, and corrected using a corresponding correction factor (see above). The resulting ratios were finally converted into a $\log_2$ base for simplicity to discriminate up- and down-regulation of a given protein in TF5015 compared with W3110. For instance, proteins showing a ratio greater than 1.0 were defined to be up-regulated, and those showing a ratio less than −1.0 to be down-regulated. Based on these criteria, ten proteins in total were identified to be up-regulated (Fig. 6), including Ppc (Table 1; no. 1), ThrA (no. 2), GltA (no. 7), ProA (no. 8), PckA (no. 14), Mdh (no. 15), IcdA (no. 20), ThrB (no. 23), AceB (no. 24), and ThrC (no. 25). In contrast, YfiD (no. 26) was found to be down-regulated. Other proteins showed no significant difference in expression levels between two strains.

As shown in Fig. 7, the developed Ab microarray exhibited a good reproducibility and reliability for the profiling of a protein expression. On a slide containing three arrays composed of quadruplicate Ab spots, the spot-to-spot variations were generally less than 7% of CV. Slides constructed on different days showed CVs lower than 20%. Based on the results taken from 32 different trials, these values are comparable with other protein microarray assays [14, 23, 24]. Thus, it is apparent that the developed Ab microarray allowed a one-shot assay for a number of target proteins in a throughput and reproducible manner.

3.5 Verification of Ab microarray assay

To validate the expression profiling of proteins by Ab microarray, we performed Western blotting and 2-DE. Expressions of the target proteins in W3110 and TF5015 were compared by measuring the thicknesses of visualized bands in Western blotting and densities of scanned spots in 2-DE (Fig. 8). The results, together with those from microarray analysis, are summarized in Table 2. In general, a 2-DE technique has been regarded as effective for analysis of diverse cellular proteins resolved depending on the p*I* and molecular weight of the protein, but is ineffective for large proteins, and those with low abundance, or embedded in a membrane [22]. Likewise, 2-DE analysis classified OppA (Table 1; no. 28) as up-regulated in TF5015, but the Ab microarray and Western blotting yielded a non-differential expression of membrane proteins OppA (no. 28) and PtsG (no. 5). Up-regulations of Ppc (no. 1) and ThrA (no. 2) were detected both in the Ab microarray and Western blotting analyses, but not in 2-DE, which seems to be due to the large size of the target proteins. These results indicate that the Ab microarray can analyze some proteins that are undetected on 2-DE. Most of the differences in protein expression between TF5015 and W3110 observed in the microarray were also identified at least in one

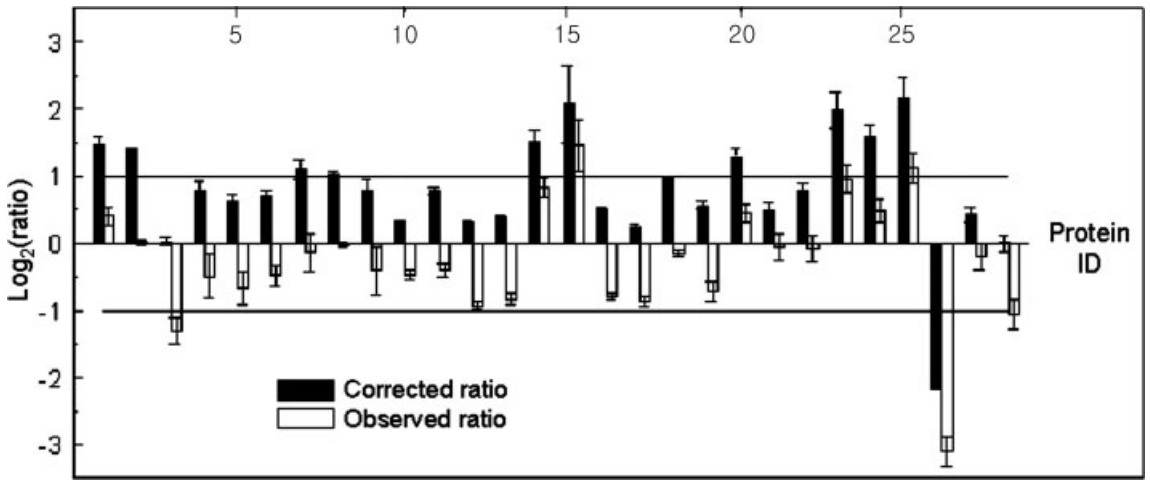

Figure 6. Profiling of protein expression. Measured ratios (open bars) were adjusted using correction factors after normalization (see text for details). The corrected ratios (closed bars) are shown as $\log_2$ values. Threshold values set for up-regulation ($\log_2 2 = 1$) and down-regulation ($\log_2 0.5 = -1$) are depicted as two horizontal lines. Data represent the average values and SDs from 32 independent measurements.

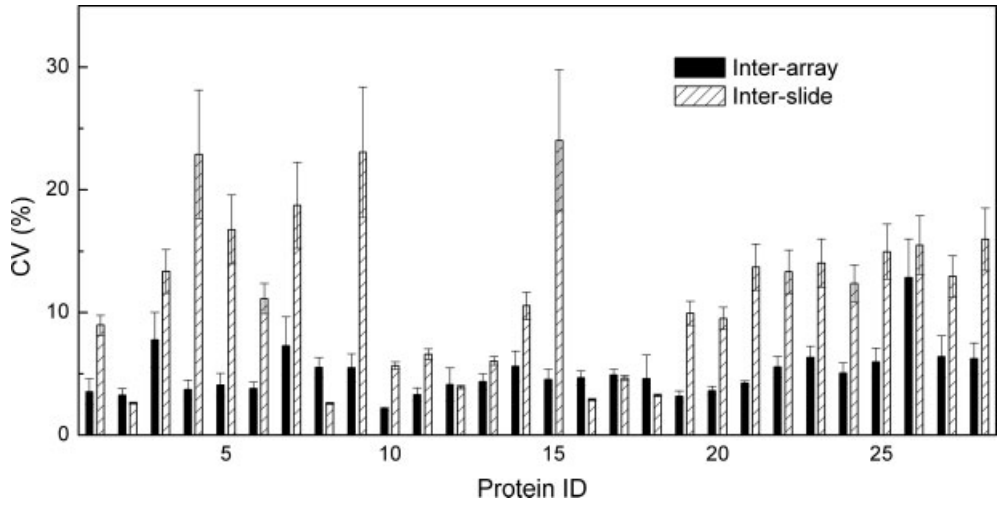

Figure 7. Inter-array and inter-slide variations of Ab microarray assays. Data indicate the average values and SDs from 32 independent measurements.

of the other analyses. For example, the up-regulation of GltA (no. 7), IcdA (no. 20), AceB (no. 24), and ThrC (no. 25), and the down-regulation of YfiD (no. 26) in TF5015, were revealed by both the Ab microarray and 2-DE analyses. Most of these differences were also detected by Western blotting analysis, except for GltA and ThrC. From these results, it is evident that the Ab microarray identifies most of the differentially expressed proteins that are detected by traditional methods.

Unlike 2-DE, the Ab microarray assays additionally classified Ppc (no. 1), ThrA (no. 2), ProA (no. 8), PckA (no. 14), Mdh (no. 15), and ThrB (no. 23) to be up-regulated in TF5015, which was also confirmed by Western blotting. In particular, ThrA (no. 2), ThrB (no. 23), and ThrC (no. 25) are key constituent enzymes of THR operon that is involved in an L-threonine biosynthetic pathway [17]. ThrA and ThrB were newly revealed to be up-regulated by the Ab microarray in addition to the ThrC that was also identified by 2-DE. Mdh and PckA are involved in the citric acid cycle, which is a critical step for synthesis of aspartate, a precursor of L-threonine. Almost all other up-regulated proteins identified by the Ab microarray and additional assays are also parts of the citric acid cycle. Some differences revealed by 2-DE and Western blotting did not appear in the Ab microarray analysis: No difference in the expression levels of AceA (no. 6) and SdhA (no. 11) between the two strains was observed in the Ab microarray assay, but these proteins were analyzed to be up-regulated in TF5015 by 2-DE or Western blotting. This result

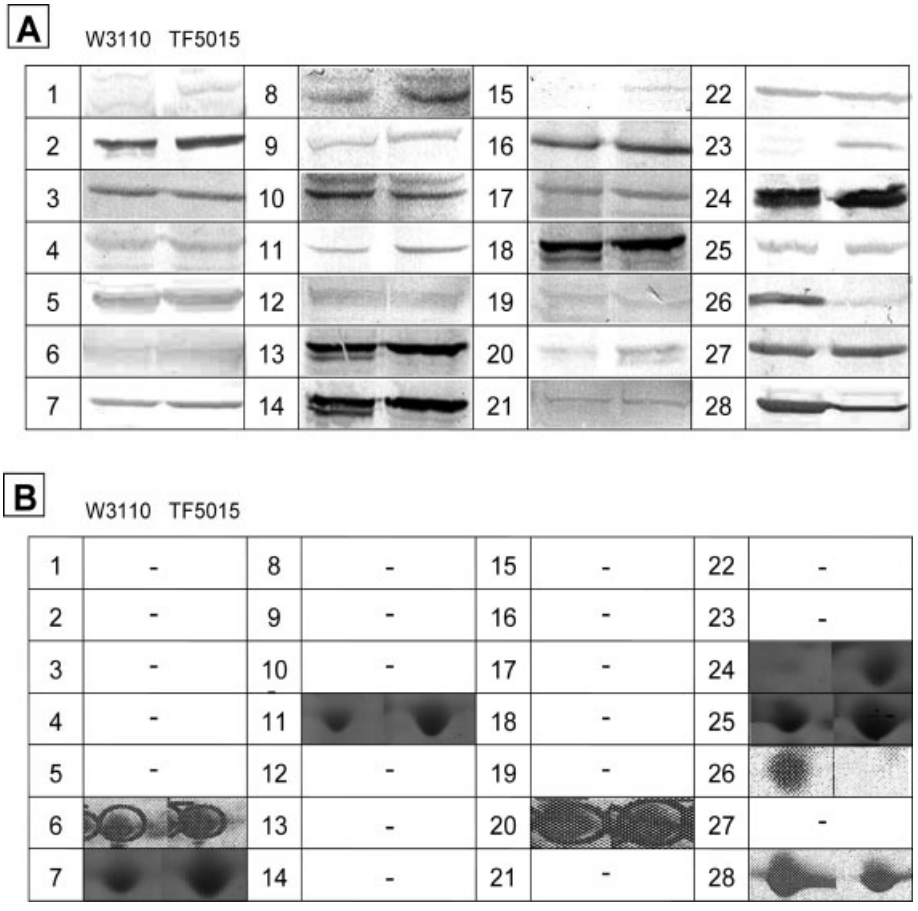

Figure 8. (A) Western blotting of 28 target proteins in W3110 (left) and TF5015 (right) lysates. (B) Spot images of 28 target proteins in W3110 (left) and TF5015 (right) lysates from 2-DE. Spots showing same densities in both strains are not displayed.

Table 2. Expression levels of target proteins in TF5015 determined by Ab microarray, Western blotting, and 2-DE.: up-regulated (▲), down-regulated (▽), and no difference (–) compared to W3110

ID no.	Protein	Ab microarray	Western blotting	2-DE
1	Ppc	▲	▲	–
2	ThrA	▲	▲	–
3	GapA	–	–	–
4	PfkA	–	–	–
5	PtsG	–	–	–
6	AceA	–	–	▲
7	GltA	▲	–	▲
8	ProA	▲	▲	–
9	Pgk	–	–	–
10	Fba	–	–	–
11	SdhA	–	▲	▲
12	Pgi	–	–	–
13	Pgm	–	–	–
14	PckA	▲	▲	–
15	Mdh	▲	▲	–
16	PfkB	–	–	–
17	DapB	–	–	–
18	Zwf	–	–	–
19	Eno	–	–	–
20	IcdA	▲	▲	▲
21	AspC	–	–	–
22	Asd	–	–	–
23	ThrB	▲	▲	–
10	Fba	–	–	–
24	AceB	▲	▲	▲
25	ThrC	▲	–	▲
26	YfiD	▽	▽	▽
27	AhpC	–	–	–
28	OppA	–	–	▽

seems to be caused by the strict threshold levels for up- and down-regulations that we employed in our analysis of the Ab microarray data. From a comparison with conventional methods including Western blotting and 2-DE, the Ab microarray seems to provide a reliable expression profiling of proteins.

4 Concluding remarks

An Ab microarray was constructed and used for the expression profiling of relevant proteins between an amino acid-overproducing mutant and its parental *E. coli* strain. For a reliable profiling, the factors affecting the performance of the Ab microarray were investigated in detail. We found that the cross-reactivity of the antibodies was the main cause of the deviation of the observed ratios from the expected ones. To offset the deviations, the correction factors for respective antibodies were introduced through statistical analysis, and used for expression profiling of proteins. As a result, of 28 tested proteins, 10 were shown to be up-regulated, and 1 down-regulated in a mutant strain. The validity of the Ab microarray data was further confirmed using Western blotting and 2-DE. The demonstrated capability of the Ab microarray-based analysis in a reliable and reproducible manner will greatly facilitate the utility of the Ab microarray in the discovery of biomarkers and the diagnosis of diseases, as well as in the expression profiling of proteins.

This work was supported by National Research Laboratory program of Ministry of Science and Technology, Nano-Medical Project of Ministry of Health and Welfare, R&D program of Fusion Strategies for Advanced Technologies of Ministry of Commerce, Industry and Energy, and Brain Korea 21 of Ministry of Education and Human Resources Development, Korea.

5 References

[1] Madoz-Gurpide, J., Wang, H., Misek, D. E., Brichory, F., Hanash, S. M., *Proteomics* 2001, *1*, 1279–1287.

[2] Andersson, O., Kozlowski, M., Garachtchenko, T., Nikoloff, C. *et al.*, *J. Proteome Res.* 2005, *4*, 758–767.

[3] Espejo, A., Cote, J., Bednarek, A., Richard, S., Bedford, M. T., *Biochem. J.* 2002, *367*, 697–702.

[4] Jahn-Schmid, B., Harwanegg, C., Hiller, R., Bohle, B. *et al.*, *Clin. Exp. Allergy* 2003, *33*, 1443–1449.

[5] Miller, J. C., Zhou, H., Kwekel, J., Cavallo, R. *et al.*, *Proteomics* 2003, *3*, 56–63.

[6] Sukhanov, S., Delafontaine, P., *Proteomics* 2005, *5*, 1274–1280.

[7] Nielsen, U. B., Cardone, M. H., Sinskey, A. J., MacBeath, G., Sorger, P. K., *Proc. Natl. Acad. Sci. USA* 2003, *100*, 9330–9335.

[8] Zhu, H., Klemic, J. F., Chang, S., Bertone, P. *et al.*, *Nat. Genet.* 2000, *26*, 283–289.

[9] Zhu, H., Bilgin, M., Bangham, R., Hall, D. *et al.*, *Science* 2001, *293*, 2101–2105.

[10] Sreekumar, A., Nyati, M. K., Varambally, S., Barrette, T. R. *et al.*, *Cancer Res.* 2001, *61*, 7585–7593.

[11] Wang, C. C., Huang, R., Sommer, M., Lisoukov, H. *et al.*, *J. Proteome Res.* 2002, *1*, 337–343.

[12] Haab, B. B., Dunham, M. J., Brown, P. O., *Genome Biol.* 2001, *2*, 1–13.

[13] Kusnezow, W., Jacob, A., Walijew, A., Diehl, F., Hoheisel, J. D., *Proteomics* 2005, *3*, 254–264.

[14] Angenendt, P., Glokler, J., Murphy, D., Lehrach, H., Cahill, D. J., *Anal. Biochem.* 2002, *309*, 253–260.

[15] de Wildt, R. M. T., Mundy, C. R., Gorick, B. D., Tomlinson, I. M., *Nat. Biotechnol.* 2000, *18*, 989–994.

[16] Angenendt, P., *Drug Discov. Today* 2005, *10*, 503–511.

[17] Lee, J. H., Lee, D. E., Lee, B. U., Kim, H. S., *J. Bacteriol.* 2003, *185*, 5442–5451.

[18] Burh, A., Flukiger, K., Erni, B., *J. Biol. Chem.* 1994, *269*, 23437–23423.

[19] Datsenko, K. A., Wanner, B. L., *Proc. Natl. Acad. Sci. USA* 2000, *97*, 6640–6645.

[20] Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G. *et al.*, *Short Protocols in Molecular Biology*, Wiley John & Sons, New York1999.

[21] Patton, W. F., Schulenberg, B., Steinberg, T. H., *Curr. Opin. Biotechnol.* 2002, *13*, 321–328.

[22] Quackenbush, J., *Nat. Genet.* 2002, *32*, 496–501.

[23] Steinhauer, C., Ressine, A., Marko-Varga, G., Laurell, T. *et al.*, *Anal. Biochem.* 2005, *341*, 204–213.

[24] Lin, Y., Huang, R., Chen, L., Lisoukov, H. *et al.*, *Proteomics* 2003, *3*, 1750–1757.

