Two-dimensional liquid chromatography protein expression mapping for differential proteomic analysis of normal and O157:H7 *Escherichia coli*

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A multidimensional chromatographic method has been applied for the differential analysis of proteins from different strains of Escherichia coli bacteria. Proteins are separated in the first dimension using chromatofocusing (CF) and further separated by nonporous reversed-phase high-performance liquid chromatography (NPS-RP-HPLC) in the second dimension. A 2-dimensional (2-D) expression map of bacterial protein content is created for virulent O157:H7 and nonvirulent E. coli strains depicting protein isoelectric point (pI) versus protein hydrophobicity. Differentially expressed proteins are further characterized using electrospray/ionization time-of-flight mass spectrometry (ESI-TOF-MS) for intact protein molecular weight (MW) determination and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) peptide mass fingerprinting for protein identification. Using this method, no significant differential protein expression is exhibited between the two O157:H7 strains examined over a pH range of 4.0–7.0, and O157:H7 strains could be distinguished from nonvirulent E. coli. Several proteins differentially expressed between O157:H7 and nonvirulent E. coli are identified as potential markers for detection and treatment of O157:H7 infection.

INTRODUCTION

Escherichia coli O157:H7 is a virulent strain of *E. coli* bacteria. A major contributor to food-borne illness, O157:H7 produces verotoxin causing hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (1–4). Culturing on specialized sorbitol-MacConkey (SMAC) agar media remains the classical method for detecting *E. coli* O157:H7. The lengthy procedure, however, exhibits only moderate specificity and routinely requires further confirmation (2,4). Complete genome sequence comparisons between *E. coli* O157:H7 strains and normal *E. coli* strains have been performed by several groups (5–7). Several O157:H7-related virulence proteins and factors are predicted from open reading frames. However, no further verification of actual gene products has been performed.

Consequently, the analysis of differential protein expression between normal and pathogenic O157:H7 *E. coli* is essential for further elucidation of the infection pathway, development of disease treatment options, and controlling environmental sources of contagion.

Proteomics provides a means to explore static genomes by analyzing the dynamics of protein expression. Traditionally, 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE) has been the method of choice for separating complex mixtures of proteins (8). However, limitations of 2-D PAGE technology have motivated proteomics researchers to hasten the development of liquid phase methods compatible with mass spectrometry (MS) for separating intact proteins. Established chromatographic theory demonstrates that 2-D system peak capacity is enhanced through utilization of orthogonal dimensions

of separation (9–11). While 2-D liquid chromatography (2-D LC) has yet to gain widespread popularity as an established proteomic tool, several 2-D liquid separation systems have demonstrated applicability to intact protein separations (12–26). The following research will discuss chromatofocusing (CF) using anion-exchange chromatography coupled with nonporous reversed-phase high-performance liquid chromatography (NPS-RP-HPLC) (27–29) for the separation of intact proteins in *E. coli* bacterial lysates. A major benefit of the 2-D LC technique discussed herein over traditional 2-D PAGE analysis is the capability to provide highly reproducible separations for interlysate comparisons among multiple protein sample pools.

CF is a protein separation technique first described by Sluyterman and co-workers (30–33). Two buffers are utilized to create a descending linear pH gradient

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where proteins elute from the column when the outlet pH equals the isoelectric point (pI) of the protein. CF has been successfully utilized as a preparative and analytical separation technique for varied simple and complex protein mixtures (27–29,34–38). A major benefit of using CF as a first dimension of separation over other chromatographic methods is that CF provides essential information about separated proteins (i.e., their pIs). Protein pI is extensively utilized in protein databases as a protein identification parameter. Each pI fraction from CF is further separated by NPS-RP-HPLC, and the entire 2-D LC separation is visualized in a convenient format similar to a gel with dimensions of pI and hydrophobicity.

In this work, two O157:H7 strains and one general *E. coli* strain are comparatively studied. Entire bacterial lysates are analyzed by 2-D LC using CF in the first dimension followed by NPS-RP-HPLC in the second dimension. Individual proteins are subsequently identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) peptide mass fingerprinting and off-line electrospray/ionization time-of-flight mass spectrometry (ESI-TOF-MS) for intact protein molecular weight (MW) determination. 2-D LC protein expression maps are generated for each bacterial sample. The 2-D maps, as well as the differential display maps, are utilized to produce an accurate comparison of protein expression in the different *E. coli* strains.

MATERIALS AND METHODS

Bacteria Culture

E. coli 88-0447 (O136STa) and the *E. coli* O157:H7 strains 96-0107 (non-motile) and 96-0112 were purchased from the Pennsylvania State University *E. coli* Reference Center, Wiley Lab, Orchard Road, University Park, PA.

Isolation and Cultivation

Using sterile forceps and aseptic technique, a tryptic soy agar (TSA) plate is streaked 3 times on a 1/3 quadrant with a sterile disposable loop. The inverted plate is placed in an incubator at 37°–41.5°C for 16–20 h. Using a

sterile disposable loop, 2 to 3 of the most isolated colonies (preferably from quadrant 3) are picked up and streaked onto the upper 1/3 quadrant of a fresh TSA plate. Using a fresh loop, the same procedure is performed to streak for isolation into 2 additional 1/3 quadrants. The inverted plate is placed in an incubator at 37°–41.5°C for 16–20 h.

One liter of *E. coli* (EC) broth [from Remel (Lenexa, KS, USA), Difco™ (BD, Franklin Lakes, NJ, USA), or equivalent] is prepared by transferring 36.6 g of medium to 1 L of deionized or distilled water and mixed until thoroughly dissolved. The media is filtered through a 0.45-μm (disposable) sterile filter. The TSA plate is removed from the incubator, and a sterile disposable loop is used to pick up a loopful of the most isolated colonies (preferably from quadrant 3) and to transfer the colonies to a bottle of media. The media is shaken vigorously and placed in incubator at 37°–41.5°C for 16–20 h. All manipula-

tions of the *E. coli* enterohemorrhagic strains 96-0107 and 96-0112 strains were performed in a Purifier® Class II Biosafety Cabinet laminar flow hood (Labconco, Kansas City, MO, USA). The strains were heat-deactivated and lysed prior to removal for CF.

Cell Pelleting

Approximately 40 mL of well mixed media is transferred to a series of 24 × 50 mL conical tubes. The tubes are capped tightly and centrifuged at full speed for 60 min using an IEC HN SII centrifuge (GMI, Albertville, MN, USA). Tubes are removed from the centrifuge, and the supernatants are discarded. Approximately 40 mL of sterile 10 mM phosphate-buffered saline (PBS) is added to each tube. Tubes are then vortex mixed to resuspend the pellet and centrifuged again at 2000× *g* for 60 min. The cell pellets are equally distributed into 12 × 2 mL centrifuge tubes using

PBS and centrifuged at $2000\times g$ for 15 min (Marathon Micro H centrifuge; Fisher Scientific, Pittsburgh, PA, USA). Cell pellets are stored at -20°C .

Sample Preparation

A 0.2- to 0.3-g cell pellet is resuspended in 0.4 mL of 50 mM Tris-HCl (pH 7.8) (Sigma, St. Louis, MO, USA) and vortex mixed vigorously. The solution is sonicated twice on ice for 30 s with a 1-min interval. The lysate is subsequently mixed with 1.6 mL of buffer containing 6 M urea, 2 M thiourea, 10% (v/v) glycerol, 50 mM Tris-HCl, 2% (w/v) n-octyl- β -D-glucopyranoside (OG), 5 mM tri(2-carboxyethyl)phosphine hydrochloride (TCEP), and 1 mM of protease inhibitor cocktail (for general use) (all from Sigma). The solution is then centrifuged at $2000\times g$ using the Marathon Micro H centrifuge for 60 min. The supernatant is removed and stored at -20°C until further use. Prior to CF, the bacterial lysate is desalted using a PD-10 Sephadex^{TM} G-25 gel filtration column (Amersham Biosciences, Piscataway, NJ, USA). The sample is then eluted from the column using the CF start buffer.

Protein Quantification

The bacterial lysates are quantified using the Bradford-based protein assay (Bio-Rad, Hercules, CA, USA). Protein concentration in each cell line varies from 14–20 mg/mL. An equal amount of total protein (4.2 mg) is loaded onto the first dimension (CF) for each cell line analyzed.

Chromatofocusing

CF is performed on an HPCF-1D column (250×2.1 mm) (Eprogen, Darien, IL, USA) using a System Gold® HPLC (Model 127S pump, Model 166 detector; Beckman Coulter, Ful-

erton, CA, USA). CF separations are monitored at UV 280 nm [absorbance units full-scale (AUFS) 0.05]. The pH gradient is generated using two buffers, an equilibration/start buffer (SB) and an elution buffer (EB). The SB is composed of 6 M urea and 25 mM Bis-Tris (Sigma) adjusted to pH 7.15 with iminodiacetic acid (Sigma). The EB is composed of 6 M urea and 10% (v/v) Polybuffer^{TM} 74 (Amersham Biosciences) adjusted to pH 4.0 also with iminodiacetic acid. The CF mobile phases are filtered through 0.20- μm nylon membrane filters (Corning, Corning, NY, USA) and degassed with helium. The removal of dissolved CO_2 is essential, as it can perturb the formation of the pH gradient.

Prior to sample CF, the HPCF column is washed with water for 1 h at 0.3 mL/min. The column is then equilibrated with SB for approximately 1 1/2 h at 0.3 mL/min until the pH of the effluent equaled the pH of the SB. A blank pH gradient is run, and then the column is re-equilibrated with SB. The pH of the mobile phase effluent is monitored online using a postdetector pH flow cell (Lazar Research Laboratories, Los An-

geles, CA, USA) with a dead volume of about 150 μL . After sample application, the baseline is allowed to stabilize before pH gradient formation. The pH gradient is established by flowing EB over the column at 0.2 mL/min. The CF separation takes approximately 1 h. Fractions are collected every 0.2 pH U using a Model SC-100 fraction collector (Beckman Coulter). Data acquisition software is written in-house using Labview recording pH, UV at 280 nm, and fraction number.

Following CF, the column is first washed with 1 M NaCl (Sigma) at 0.5 mL/min until the baseline stabilizes and then rinsed with 100% 2-propanol at 0.2 mL/min until the baseline stabilizes. The column is then rinsed with water overnight to remove all residual salts. CF columns should be stored in 2-propanol (Sigma).

Nonporous Reversed-Phase HPLC

A System Gold HPLC is also used for the second dimension separation of each collected CF fraction. The instrument is equipped with a Model 127S solvent module and a Model 166

Figure 1. Two-dimensional liquid chromatography (2-D LC) protein expression map of *Escherichia coli* bacterial lysate. The x-axis is in isoelectric point (pI) U from pH 4.0 to 7.0 in 0.2-U increments, and the y-axis displays increasing hydrophobicity (%B).

detector with a 15- μ L analytical flow cell. All second dimension separations are monitored using a deuterium lamp at 214 nm (AUFS 1.00). Separations are performed at a 1.0 mL/min flow rate using a (33 $\times$ 4.6 mm) RP-HPLC column packed with 1.5- μ m nonporous ODS-III (C18) silica beads (Eprogen). The NPS-RP-HPLC separation of bacterial proteins in CF fractions is performed using gradient elution with two solvents [solvent A: MilliQ® water (Millipore, Billerica, MA, USA) with 0.1% (v/v) trifluoroacetic acid (TFA) (Sigma); solvent B: acetonitrile (ACN) (Sigma) with 0.1% (v/v) TFA]. Mobile phases are sonicated, and helium sparged. The column temperature is maintained at 60°C using a Model 7971 column heater (Jones Chromatography, Resolution Systems, Holland, MI, USA). The gradient profile used for solvent B on the nonporous columns is as follows: 5%–25% B in 1 min, 25%–31% B in 1 min, 31%–37% B in 8 min, 37%–41% B in 8 min, 41%–67% B in 8 min, 67%–100% B in 2 min, 100% B for 1 min, and 100%–5% B in 1 min.

Fractions from the HPLC eluent are collected using a semi-automated in-house program using a Model SC-100 fraction collector. Collected peak fractions are divided for both MALDI-TOF-MS peptide mass fingerprinting analysis and intact MW analysis using ESI-TOF-MS.

Electrospray/Ionization TOF-MS

ESI-TOF-MS analyses of collected protein peak fractions are performed on a Micromass LCT™ workstation (Waters, Milford, MA, USA). Ions are generated from a z-spray source with a desolvation gas temperature of 150°C at a flow rate of approximately 450 L/h. The source is held at 100°C. The capillary voltage is +2800 V, the sample cone is +45 V, the extraction cone is +2 V, and the hexapole RF is +750 V with a DC offset of +3 V. The second hexapole is biased to -3 V, and the detector is held at -2750 V. External calibration is performed by direct infusion of a standard NaI/CsI (Sigma) solution. Fractions are infused into the source using a syringe pump (Harvard Apparatus, Holliston, MA, USA) at a flow rate of 30–50 μ L/min.

Data is processed using Micromass MassLynx™ v3.4 software (Waters), and protein multiply charged umbrellas are deconvoluted using Micromass MaxEnt® 1 software (Waters). Deconvolution is performed using a mass range of 5–85 kDa, 1 Da resolution, 0.75 Da peak width, and a 65% peak height value.

Tryptic Digestion of Nonporous Reversed-Phase HPLC Fractions

Fractions from NPS-RP-HPLC are reduced to a volume of 80 μ L using a vacuum concentrator (Centrivap Concentrator; Labconco). Then, 10 μ L of 1 M ammonium bicarbonate (Sigma), 5 μ L of 10 mM dithiothreitol (DTT) (Sigma), and 0.25 μ g of N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-modified porcine trypsin (Promega, Madison, WI, USA) are added to each fraction. The fractions are vortex mixed and incubated at 37°C for 12 h. Following incubation, 50 μ L of fresh 0.1% (v/v) TFA is added to each tube to halt digestion.

MALDI-TOF-MS Sample Preparation and Data Acquisition

Tryptic digests are desalted and concentrated using 2- μ m C18 Zip-Tips (Millipore) eluted into 10 μ L of 60% (v/v) ACN/0.1% (v/v) TFA. An α -cyanohydroxycinnamic acid (α -CHCA; Sigma) matrix solution is prepared that contains the following internal standards: angiotensin I, adrenocorticotrophic hormone (ACTH) fragment 1–17, and ACTH fragment 18–39 (all from Sigma). Internal standards permit sample spectra to be calibrated to a mass accuracy of 10–50 parts per million (ppm). A saturated solution of α -CHCA is first prepared in 100% ACN, diluted 1:4 with 60% (v/v) ACN/1% (v/v) TFA, and then internal standard peptides are added such that the concentration of these peptides in each sample spot will be approximately 50 fmol. Matrix solution (0.5 μ L) and 0.5 μ L of sample are spotted on the MALDI plate using a two-layer method.

The Micromass TofSpec-2E™ (Waters) is used in reflectron mode with positive polarity at an operat-

ing voltage of 20 kV and a reflectron voltage of 24.5 kV. The pulse voltage used for delayed extraction is between 2000–2200 V at a 520 ns delay time. The matrix suppression setting was set at the maximum of 500 V. The sampling rate was 2 GHz, and each spectrum recorded was an average of 75–100 laser shots. The nitrogen laser (UV, 337 nm) was used at 50% laser coarse and 35–40 laser fine control.

Spectra are calibrated using the internal calibrants, and monoisotopic peak lists are generated and submitted to MS-Fit (<http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>) (using the SwissProt protein databases) for protein identification. Protein identifications are considered confident matches when meeting the following criteria: (i) ranked in the top three database hits; (ii) sequence coverage is >20%; and (iii) average mass error is <50 ppm.

Chemicals

All reagents are used without any further purification and are of the highest quality commercially available. All solvents are HPLC-grade, and water is purified using a MilliQ water filtration system (Millipore).

2-D Liquid Chromatography Protein Expression Mapping Software

2-D LC separations are displayed using ProteoVue™ software that was developed in cooperation between the University of Michigan and Eprogen. The ProteoVue software converts the chromatographic intensities from the NPS-RP-HPLC separation of each pH fraction from CF into a band intensity format. This produces a highly detailed map with the dimensions of hydrophobicity and pI. The 2-D LC maps can be viewed in either gray-scale or one of several colored formats where either hue or color intensity is proportional to the relative intensity of each chromatographic peak. Another software program, DeltaVue™ (Eprogen), is used for the differential analysis of corresponding fractions from two different sample sets. A differential map is achieved by point-to-point subtraction and is viewed between the two original sample sets.

A. Strain 88-0447 versus strain 96-0107

B. Strain 88-0447 versus strain 96-0112

C. Strain 96-0107 versus strain 96-0112

Figure 2. Differential display protein expression maps for all three bacterial strains. Differentially expressed proteins are viewed in the center map of each figure panel. The lanes in the center map are obtained by point-to-point subtraction of corresponding data from the maps on either side. (A) Strain 88-0447 (red) versus strain 96-0107 (green). (B) Strain 88-0447 (red) versus strain 96-0112 (green). (C) Strain 96-0107 (red) versus strain 96-0112 (green).

Figure 3. Two-dimensional (2-D) differential maps of selected isoelectric point (pI) fractions. (A) Chromatofocusing (CF) pH fraction 5.0–5.2; (B) CF pH fractions 5.2–5.4; (C) CF pH fractions 5.4–5.6; (D) CF pH fractions 6.2–6.4. Normal *Escherichia coli* is shown in the red lane versus O157:H7 *E. coli* in the green lane. The center lane depicts proteins differentially expressed between the two bacterial strains. Protein bands identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) are labeled.

Table 1. Proteins Identified from 2-Dimensional Liquid Chromatography Fractions

Protein Name	Sequence Covered (%)	MW/pI	Accession No.	Actual MW	Change
Peptidyl-prolyl <i>cis-trans</i> isomerase B (PPIaseB) (rotamase B)	42	18154/5.5	P23869	18151.82	A
Spermidine/putrescine-binding periplasmic protein precursor (SPBP)	41	38867/5.2	P23861	36476.035	AA
Cysteine synthase A (o-acetylserine sulfhydrylase A) (o-acetylserine (thiol)-lyase A) (CSase A) (sulfate starvation-induced protein 5) (SSI5)	34	34490/5.8	P11096	34354.715	A
PTS system, mannose-specific IIAB component (EIIAB-Man) (mannose-permease IIAB component) (phosphotransferase enzyme II, AB component) (EIII-Man)	33	35048/5.7	P08186	34915.578	AA
DNA protection during starvation protein	64	18695/5.7	P27430	18563.486	AA
Methylglyoxal synthase (MGS)	41	16919/6.1	P37066	16880.43	AA
DNA K suppressor protein	28	17528/5.1	P18274	17524.36	AB
Protein yfiD	58	14284/5.1	P33633	14284.5	B
Shikimate kinase I (SKI)	42	19538/5.3	P24167	19406.79	B
Chaperone protein hscB (Hsc20)	60	20138/5.0	P36540	20133.04	AB
Protoporphyrinogen oxidase (PPO)	25	21226/9.7	P27863	21245.8	B
Inorganic pyrophosphatase (pyrophosphate phospho-hydrolase) (PPase)	35	19704/5.0	P17288	19597.57	B
Protein yifE	52	13134/6.1	P27827	13001.39	BB
DNA-directed RNA polymerase omega chain (transcriptase omega chain) (RNA polymerase omega subunit)	75	10237/4.9	P08374	10105.06	B
Protein yfiA	47	12785/6.2	P11285	12652.9	B
Ornithine carbamoyltransferase chain F (OTCase-2)	30	36827/5.6	P06960	36480.27	AB
DNA-binding protein H-NS (histone-like protein HLP-II) (protein H1) (protein B1)	64	15540/5.4	P08936 M	15450.61	B
Glyceraldehyde 3-phosphate dehydrogenase A (GAPDH-A)	57	35533/6.6	P06977	35195.15	BB
Protein ygiN	40	11533/5.8	P40718	11529.18	B
Hypothetical protein yaeB	25	26363/7.9	P28634	26095.47	BB
HIT-like protein ycfF	81	13241/5.7	P36950	13110.36	BB
Curved DNA-binding protein	32	34455/6.3	P36659	*	B
UDP-glucose 4-epimerase (galactowaldenase) (UDP-galactose 4-epimerase)	26	37265/5.9	P09147	37394.28	B
Modulator of drug activity B	40	21891/5.8	P40717	21801.32	B
Unknown protein from 2-D PAGE (spots PR25/LM16/2D_000LR3)	63	15935/6.0	P39177	15936.82	B
Transcription termination factor rho	31	47005/6.8	P03002	*	BB
Autoinducer-2 production protein luxS (AI-2 synthesis protein)	49	19416/5.2	P45578	*	AA
Molybdenum cofactor biosynthesis protein B	47	18665/5.7	P30746		AA
Hypothetical protein yccJ	81	8525/4.7	P46131	*	AA
Delta-aminolevulinic acid dehydratase (porphobilinogen synthase) (ALAD) (ALADH)	30	35625/5.2	P15002	*	A
Trimethylamine-N-oxide reductase 2 precursor (TMAO reductase 2) (trimethylamine oxidase 2)	22	88965/6.8	P46923	*	A
Exu regulon transcriptional regulator	43	29836/5.4	P42608	*	A
TRAM protein	38	15581/5.4	P71198	*	AB

*MW, molecular weight was not determined; A, overexpressed in general *Escherichia coli*; B, overexpressed in O157:H7; AA, only present in general *E. coli*; BB, only present in O157:H7; AB, present in both equally. pI, isoelectric point.

RESULTS

Presuming that the virulent nature of *E. coli* O157:H7 is reflected in changes in bacterial protein expression, lysates of both normal and O157:H7 *E. coli* bacteria are utilized to analyze differential protein expression. Comparative analysis is performed using one normal strain (88-0447) and two virulent O157:H7 strains (96-0107 and 96-0112). Proteins from bacterial lysates are first fractionated according to their pI using CF. Subsequently, each pI fraction is further separated using NPS-RP-HPLC. A 2-D LC protein expression map is then generated using ProteoVue software, which conveniently presents the pI of the eluted protein from CF on the x-axis and the retention time, or hydrophobicity, from NPS-RP-HPLC of each protein peak on the y-axis. Figure 1 displays the 2-D LC expression map of virulent 96-0107 *E. coli*. A total protein load of 4.2 mg is initially loaded onto the first dimension

CF column. CF columns used in these experiments can tolerate sample loads up to 5 mg and still produce high-resolution pH separations. CF separations are monitored at 280 nm instead of 214 nm as in NPS-RP-HPLC separations in order to avoid interference from elution buffer contents. The use of Polybuffer in the CF elution buffer creates an even linear pH gradient. Polybuffer is available for pH 7.0–4.0 and pH 9.0–6.0 separations. Polybuffer 74 is employed in these experiments, as the majority of proteins in the bacterial lysates migrated between pH 4.0 and 7.0 in 2-D PAGE experiments (data not shown).

Figure 2 presents 2-D LC differential display protein expression maps generated using DeltaVue for all three bacterial strains. Initial observation reveals striking similarities between the maps of the two O157:H7 strains (Figure 2C), 96-0107, and 96-0112. Furthermore, the differential maps of both O157:H7 strains versus the normal *E. coli* strain (88-0447) exhibit similar differential expression (Figure 2, A and B). Equivalent amounts of total protein (4.2 mg) from each bacterial strain are used to create the differential maps in Figure 2. Each bacterial lysate is pooled into 15 fractions of 0.2 pH U from pH 4.0 to 7.0 and then separated by hydrophobicity using NPS-RP-HPLC. The DeltaVue maps compare HPLC chromatograms from different samples with the same pI ranges. HPLC data sets from two samples, one sample represented in red and the other in green, are transformed into gel-like images with a band and lane format, where band color intensity mirrors peak intensity from RP-HPLC. Bands on the differential image found in the center of each DeltaVue map are formed by point-to-point subtraction of corresponding lane data. In the differential map, the absence of bands indicates no differential protein expression between the samples, and the presence of colored bands suggests that protein expression of the particular band is higher in the sample of the same band color. While several differential bands are observed between 88-0447 and 96-0107 as well as between 88-0447 and 96-0112 over the pH range from 5.0 to 6.4, no strong bands appear on the differential maps between pH 4.0 and 5.0, suggesting no significant differential protein expres-

sion among these strains in this acidic range. Furthermore, the differential map between 88-0447 and 96-0107 exhibits the same pattern with minor differences in relative intensity as the differential map between 88-0447 and 96-0112. Consequently, few strong bands appear on the differential map between the two O157:H7 strains, 96-0107 and 96-0112. Additionally, differential maps reveal some higher abundance proteins present in the normal strain (88-0447) that disappear in the O157:H7 strains (96-0107 and 96-0112) and vice versa. Such proteins are investigated further as they can potentially serve as markers for distinguishing normal *E. coli* from virulent O157:H7 *E. coli*.

Since *E. coli* has been well studied, only potential proteins in four pI ranges (5.0–5.2, 5.2–5.4, 5.4–5.6, and 6.2–6.4) are targeted for further characterization by ESI-TOF-MS and MALDI-TOF-MS. These fractions are selected owing to clear differences displayed in differential maps between the O157:H7 strains and the normal *E. coli* strain. Figure 3 shows the comparative maps between the four selected pI fractions. Since the two O157:H7 strains exhibit similar overall protein expression, only one of the O157:H7 strains is compared with fractions from the normal strain (88-0447). Proteins identified by MALDI-TOF-MS peptide mass fingerprinting are labeled in Figure 3, and corresponding ESI-TOF-MS intact molecular weight determination and differential expression information are summarized in Table 1. Since HPLC is performed on fractions with identical pIs under the same separation conditions, differences in the second dimension relative UV intensity can be correlated to differential expression level for specific protein peaks (bands). Table 1 reveals six proteins only expressed in the normal *E. coli* strain and five proteins only associated with O157:H7 *E. coli* strains. Additionally, Table 1 catalogs twelve proteins present in O157:H7 and five proteins present in normal *E. coli* identified as overexpressed and three proteins present in the strains at equivalent concentrations. None of the identified proteins overexpressed in O157:H7 *E. coli* are found to be virulence-specific as compared with genome predictions.

However, the functions of some identified proteins, such as protein yfiE and yfiA, are not defined in the database searched. Furthermore, not all differentially expressed proteins are present at concentrations high enough to permit definitive protein identification.

Proteins expressed in only one bacteria strain can be potentially used as markers to differentiate the bacteria. Even without protein identification, a 2-D LC expression map pattern can be used independently as a fingerprint for *E. coli* O157:H7 detection. The 2-D LC method described herein is a powerful tool for pattern comparison between different protein pools.

DISCUSSION

A multidimensional chromatographic technique has been applied to the separation and comparison of *E. coli* bacteria lysates using CF coupled with NPS-RP-HPLC. The method provides a convenient 2-D map of protein expression with proteins categorized by both pI and hydrophobicity. The 2-D LC method offers a significantly higher loadability than conventional techniques while maintaining a high degree of resolution. While 0.2 pH U fractions were collected in this separation, CF is capable of 0.05 pH U fractionation. Higher loadability greatly enhances the detection of lower abundance proteins and is a necessity for multidimensional techniques aimed at separating complex samples. The superior fractionation achieved in both separation dimensions results in a higher peak capacity than conventional 2-D PAGE expression mapping. Furthermore, the exact protein pI and hydrophobicity [measured in %B (ACN)] values obtained uniquely identify a particular protein component and permit its collection for further characterization.

The use of 2-D LC mapping for analyzing protein expression has many advantages. The 2-D LC map generated by coupling CF with NPS-RP-HPLC creates a user-friendly map of protein expression that is easy to interpret and manipulate. The ProteoVue software allows the user to zoom in and out of regions of interest in order to view small details in the UV profiles. Furthermore,

DeltaVue software provides a convenient means to display and analyze differential expression between two different sample pools. Employment of liquid phase separations facilitates ease of automation of the entire 2-D process with minimal user interference. In addition, liquid phase separations support the collection of protein peaks/bands of interest for further characterization by MALDI-TOF-MS peptide mass fingerprinting, MS/MS peptide sequencing, ESI-TOF-MS intact protein MW analysis, or other methods.

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