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From Proteome to Genome for Functional Characterization of pH-Dependent Outer Membrane Proteins in *Escherichia coli*

Lina Wu, Xiangmin Lin, and Xuanxian Peng*

Center for Proteomics, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China

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Proteomic technology is very powerful in identification of differentially expressed proteins. However, how to identify key proteins and distinguish them from others has been a question to be solved in functional proteomics. Utilizing 2-D gel based proteomic approach, we identified 11 differentially expressed outer membrane (OM) proteins involved in *E. coli*'s response to pH change. The protein expression changes were validated by Western blotting. The function and roles of the differentially expressed proteins were further characterized using genetically modified strains with gene deletion of these altered OM proteins and gene complementation or overexpression approach. Among the differentially expressed proteins identified, OstA, TolC, OmpT, OmpP OmpC, Trak, OmpX, Dps, LamB, Tsx, FadL, OmpW, and OmpF were characterized as pH-related OM proteins. Out of these OM proteins, TolC, OmpC, OmpX, and LamB may play critical roles in pH-regulation in *E. coli*. Using death-rescuing assay developed in house, we found that OmpC, LamB, FadL, OmpX, OmpW, and OmpF, LamB, FadL, and OmpW functioned in a TolC-independent pathway, whereas OmpF, Tsx and OmpC, OmpX, and Tsx might share the same pathway with TolC at the extreme acid or base condition. The information obtained from this study provides novel insight into mechanisms of pH response in *E. coli*. Our results also demonstrate the importance and efficiency of functional characterization of differentially expressed proteins at different molecular levels in identification of key target proteins and pathways involved in *E. coli*'s response to pH change.

Keywords: outer membrane proteins • pH • proteomics • genomics • genetically modified strains • *Escherichia coli*

Introduction

Bacteria are able to change their gene and protein expression rapidly and efficiently in response to environmental signals for their survival in various and rapidly changing environments.^{1–7} pH is one of the environmental signals. The survival of bacteria under their unexpected acidic and basic environments in which growth is inhibited is a question of great interest to current bacterial study due to the contribution of pH to pathogenesis of enteric bacteria. These bacteria grow best at neutral pH, but they also can grow in moderately acidic or basic conditions, with a positive or negative transmembrane pH difference. By regulating gene and protein expression, they can survive in low pH of stomach with gastric acid secretion and high pH of intestine with pancreatic secretion.^{7,8} For example, *Escherichia coli*, one of the enteric bacteria, normally grows over a range of environmental pH 4.5–9 but can always maintain its internal pH between 7.4 and 7.9 during its growth.⁹ This is largely dependent on its strong ability in regulation of its internal pH

value.^{10,11} However, little is known about mechanisms underlying its survival ability under extremely acidic and basic conditions.

Previous publications have indicated that expression of several envelope, periplasmic, and cytoplasmic components is pH-dependent.^{1,12–14} The proteins of envelope and periplasm are more vulnerable than those of cytoplasm when the enteric bacteria are exposed to the harsh environmental conditions encountered in the low and high pH of the mammalian stomach and intestine, respectively. For cytoplasmic components, virulent factors are usually target proteins. Elevation of these proteins is documented in low pH.^{15–18} They include ToxR–ToxT virulence regulon of *Vibrio cholerae*,¹⁵ *phoP-phoQ* regulon of *Salmonella enterica*,¹⁶ pH 6 antigen of *Yersinia pestis*,¹⁷ and RpoS and regulators of arginine decarboxylase (adi) of *E. coli* O157:H7.^{18,19} For envelope and periplasmic components, porins and their associated proteins play important roles in the response. Upregulation of OmpC and downregulation of OmpF are detected when *E. coli* survives in a medium with low pH. Neither OmpC nor OmpF is detectable in an *ompR* mutant at acid condition, and the expression of *ompC*- and *ompF-lacZ* fusion genes reduces in an *envZ* mutant.²⁰ Further proteomic analysis of whole cell lysates reveals upregulation of OmpX and OmpA at both acidic and

* To whom correspondence should be addressed. Xuan-Xian Peng, State Key Laboratory for Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, People's Republic of China. Tel: +86-20-3145-2846. E-mail: pxuan@sysu.edu.cn; wangpeng@xmu.edu.cn.

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Table 1. Strains of *E. coli* K-12 Used in This Study

strain	relevant characteristic(s)	source or reference
<i>E. coli</i> K12 K99+	wild type	Institute of Microbiology, Chinese Academy of Sciences, Beijing, China
BW25113	<i>lacI rrnBT14ΔlacZWJ16hsdR514 ΔaraBADAH33 ΔrhaBADLD78</i>	Nara Institute of Science and Technology
△ <i>tolC</i>	BW25113, chromosomal deletion of <i>tolC</i>	This study
△ <i>ompC</i>	BW25113, chromosomal deletion of <i>ompC</i>	This study
△ <i>ompW</i>	BW25113, chromosomal deletion of <i>ompW</i>	This study
△ <i>ompT</i>	BW25113, chromosomal deletion of <i>ompT</i>	Nara Institute of Science and Technology
△ <i>ompX</i>	BW25113, chromosomal deletion of <i>ompX</i>	Nara Institute of Science and Technology
△ <i>dps</i>	BW25113, chromosomal deletion of <i>dps</i>	Nara Institute of Science and Technology
△ <i>lamB</i>	BW25113, chromosomal deletion of <i>lamB</i>	Nara Institute of Science and Technology
△ <i>tsx</i>	BW25113, chromosomal deletion of <i>tsx</i>	Nara Institute of Science and Technology
△ <i>fadL</i>	BW25113, chromosomal deletion of <i>fadL</i>	Nara Institute of Science and Technology
△ <i>ompF</i>	BW25113, chromosomal deletion of <i>ompF</i>	Nara Institute of Science and Technology
+ <i>tolC</i>	△ <i>tolC</i> complementation by <i>tolC</i> of <i>E. coli</i> K-12	This study
+ <i>ompC</i>	△ <i>ompC</i> complementation by <i>ompC</i> of <i>E. coli</i> K-12	This study
+ <i>lamB</i>	△ <i>lamB</i> complementation by <i>lamB</i> of <i>E. coli</i> K-12	This study
* <i>ompC</i>	△ <i>tolC</i> harboring pLLP- <i>ompA-ompC</i>	This study
* <i>ompX</i> ,	△ <i>tolC</i> harboring pLLP- <i>ompA-ompX</i>	This study
* <i>lamB</i>	△ <i>tolC</i> harboring pLLP- <i>ompA-lamB</i>	This study
* <i>tsx</i>	△ <i>tolC</i> harboring pLLP- <i>ompA-tsx</i>	This study
* <i>fadL</i>	△ <i>tolC</i> harboring pLLP- <i>ompA-fadL</i>	This study
* <i>ompW</i>	△ <i>tolC</i> harboring pLLP- <i>ompA-ompW</i>	This study
* <i>ompF</i>	△ <i>tolC</i> harboring pLLP- <i>ompA-ompF</i>	This study

basic conditions, and upregulation of YdiY, TolC, and YfiD at an acidic condition, and MalE and MalB at a basic condition. On the other hand, the results obtained from DNA microarray analysis indicate a up-regulation of *fadL* and *malE*, and down-regulation of *ompT*, *tsx*, and *lamB* at a basic condition, down-regulation of *ompC* and *lamB* at an acidic condition.²¹ In general, less is known about base-dependent proteins than acid-dependent ones in response to pH. To identify more differential expressed proteins in response to pH, especially key proteins involved in the pH response, we performed a parallel investigation of altered subproteome and subgenome using *E. coli* cultured in mediums with extreme acid or base pH values. OM proteins involved in pH regulation were identified. The importance of these proteins involved in pH-response of *E. coli* was functionally characterized. Furthermore, the OM protein-mediated pH-response pathways in *E. coli* were classified using a novel death-rescuing approach established in the present study.

Materials and Methods

Strains and Growth Conditions. The bacterial strains used in this work are described in Table 1. Growth medium contained potassium-modified Luria broth (LBK) (10 g of tryptone, 5 g of yeast extract, 7.45 g of KCl per liter) was adjusted for pH using 100 mM buffer KOH instead of NaOH to avoid inhibition of growth by Na⁺ at high pH.²² The buffers used to adjust pH of the culture mediums were as follows: pH 4.5 and pH 4.9, homopiperazine-*N,N'*-bis-2(ethanesulfonic acid) (HOMOPIPES); pH 6.0, 2-(*N*-morpholino)ethanesulfonic acid (MES); pH 7.4, 3-(*N*-morpholino)propanesulfonic acid (MOPS); pH 9, pH 9.5, and pH 9.8, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO).^{2,6,23,24} All the buffers were obtained from Research Organics or Sigma. For all the cultures, the pH was measured after growth to ensure that the values were maintained at ±0.1 pH unit of the pH of the original medium. *E. coli* K12 BW25113 was grown overnight in unbuffered LBK, then diluted 500-fold in fresh

buffered medium and grown to an optical density at 600 nm (OD600) of 0.2 at 37 °C in baffled flasks with rotary aeration. Optical density readings (OD600) were obtained using a Spec-tramax Plus microtiter plate spectrophotometer (Molecular Devices).

Extraction of OM Proteins. OM proteins of *E. coli* K12 were prepared according to a procedure described previously.²⁵ Briefly, bacterial cells were harvested by centrifugation at 4000 g for 15 min at 4 °C. The bacterial cells were washed in 40 mL sterile saline three times and then resuspended in 10 mL of sonication buffer [50 mM Tris-HCl (pH 7.4)]. The cells were disrupted by intermittent sonic oscillation for a total of 15 min at 50% power with intervals of 9 s on ice. Unbroken cells and cellular debris were removed by centrifugation at 5000 g for 20 min, and the supernatant was collected and was further centrifuged at 100 000 g for 40 min at 4 °C. The pellet was resuspended in 10 mL of 2% w/v sodium lauryl sarcosinate (Sigma) and incubated at room temperature for 1 h. The solution was centrifuged at 100 000 g for 40 min at 4 °C. The resulting pellet was resuspended in sonication buffer and stored at -80 °C until use. The concentration of the OM proteins in the final preparation was determined using the Bradford method.

2-DE and Mass Spectrometric Analysis. 2-DE was performed according to a procedure described previously.²⁵ Briefly, OM protein extracts containing 15 µg of proteins were dissolved in a solution (8 M urea, 2 M thiourea, 4% CHAPS, and 80 mM DTT). IEF was carried out using pH 3–9.5 carrier ampholyte for 8000 Vh. After being equilibrated for 15 min, the IEF gels were transferred to the second dimension electrophoresis using 12% acrylamide gel. The reference condition was defined as growth at pH 7.0, while cultures at pH 6.0 and 9.1 were used as comparative groups. For each growth condition, spot densities were obtained from three gel images from independently grown cultures. To compare spot quantities between different growth conditions, the 2-DE gels were stained with Coomassie brilliant blue (CBB) R-250. Subsequently, gels were

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scanned in an AGFA white-light scanner at a resolution of 400 by 200 mm and the raw images were processed using the 2-D software Melanie 4.0 (Swiss Institute of Bioinformatics, Geneva, Switzerland). Each spot intensity volume was processed by background subtraction and total spot volume normalization; the resulting spot volume percentage was used for comparison. Mass spectrometric analysis was carried out according to a procedure described previously.²⁵

SDS-PAGE and Immunoblotting. SDS-PAGE analysis of samples was performed in 12% (w/v) polyacrylamide gels. These samples included OM preparation and whole cell (about 10^7 cell equivalents) lysates of *E. coli* K12 BW25113 growing in different pH, pH 4.5, 6, 7.4, 9.0, and 9.5. These samples were separately quantified and solubilized at 100 °C for 5min in sample buffer containing 2% (w/v) SDS and 5% (v/v) beta-mercaptoethanol. Following electrophoresis, resolved bands were visualized by CBB R-250 staining. Western blotting was performed using rabbit antisera to respective OM proteins. These antisera were commercially obtained from Wenta Bio Sci Tech Corp (Ji'an, China) and their specificity was validated before use. Proteins in gels were transferred to nitrocellulose membranes by electrophoreses, followed by immunodetection of OM proteins using the rabbit antisera as the primary antibodies and peroxidase-conjugated goat antirabbit IgG as the secondary antibody. Subsequently, the membranes were scanned in an AGFA white-light scanner at a resolution of 400 by 200 mm and the raw images were processed using the 1-D software phoretix1-D. Following background subtraction and band detection, the membrane patterns were matched to each other by visual comparison, and then these bands were compared based on their volume to the band of pH 7.4.

Construction of *ΔompW*, *ΔompC*, and *ΔtolC*. All of the mutants used are derivatives of *E. coli* K12 BW25113 [*lacI_{P4000(laciQ)} rrrB3_lacZ4787 hsdR514_(araBAD)567_rhaBAD568 rph-1*].²⁶ *ΔompT*, *ΔompX*, *Δdps*, *ΔlamB*, *Δtsx*, *ΔfadL*, and *ΔompF* were kindly provided by Nara Institute of Science and Technology.²⁷ *ΔompW*, *ΔompC*, and *ΔtolC* were constructed in our laboratory using a standard chromosomal gene disruption protocol.²⁸ Briefly, PCR products were generated using a pair of long (usually 56- to 60-nucleotide [nt]) primers and special template plasmids bearing a resistance marker flanked by FLP recombinase target sites. These oligonucleotides included 20 nt for priming on template plasmids and 36- to 40-nt homologous extensions for targeting recombination events to the corresponding loci on the *E. coli* chromosome. The PCR products were then introduced into cells expressing the phage λ Red recombinase, which permitted recombination in short homologous regions. Recombinants were then verified in a series of PCR tests in which locus-specific test primers were used, as described elsewhere.²⁸ The test primers flanked the deleted region and usually were 200 nt or more from the homologous extension regions.

Complementation of Gene Deletion Mutants. Complementation experiment was based on previously described procedures.³ By using the appropriate primer pairs designed, genes *tolC*, *ompC*, and *lamB* were amplified from *E. coli* K12 BW25113, introducing *Bam*H1 restriction sites. The amplified products were cloned into the *Bam*H1 sites in pACYC184 (New England Biolabs), leaving the genes under the control of the chlormycetin resistance gene promoter, thus generating *ptolC*, *pompC*, and *plamB*. They were separately electrotransformed into electrocompetents of *E. coli* K12 BW25113 mutants *ΔtolC*,

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ΔompC, and *ΔlamB*, thus generating *+tolC*, *+ompC*, and *+lamB*, respectively.

Construction of Gene Expression Plasmids and Their Overexpression in Host Strain *ΔtolC* (Death-Rescuing). Standard molecular cloning techniques were used to clone target genes to vector PLLP-*ompA*, generating *ppompC*, *ppompX*, *pplamB*, *pptsx*, *ppfadL*, *ppompW*, and *ppompF*. This vector is accompanied with an *ompA* signal which can secrete the expression protein to the periplasmic space. These *ppompC*, *ppompX*, *pplamB*, *pptsx*, *ppfadL*, *ppompW*, and *ppompF* were separately electrotransformed into *ΔtolC*, generating **ompC*, **ompX*, **lamB*, **tsx*, **fadL*, **ompW*, and **ompF*. PLLP-*ompA* was electrotransformed in to *ΔtolC*, generating **pp* as a control. The single colony of **pp*, **ompC*, **ompX*, **lamB*, **tsx*, **fadL*, **ompW*, and **ompF* was grown in 5 mL LB medium at 37 °C till the optical density (OD600) of the cultures at 0.5. Induction of the expression of *ompC*, *ompX*, *lamB*, *tsx*, *fadL*, *ompW*, and *ompF* was obtained by adding IPTG to a final 68 µg/mL concentration for 3 h at 30 °C. **pp*, **ompC*, **ompX*, **lamB*, **tsx*, **fadL*, **ompW*, and **ompF* cultures were separately diluted 1:200 to the 20mL buffered LBK medium of pH 4.5 and 9.8 for survival experiment.

Measurement of Bacterial Growth. *E. coli* K12 BW25113 and its mutants were grown in 5 mL of unbuffered LBK medium overnight. The initial optical density (OD600) of the culture was 0.10–0.12, which corresponds to approximately 10^7 cells per mL. Fresh overnight cultures were separately diluted 1:200 to the 200 mL buffered LBK medium of pH 4.5, 4.9, 6.0, 7.4, 9.0, 9.5, and 9.8, and then incubated at 37 °C with shaking at 200 rpm. The growth curves were measured at 0, 2, 4, 6, 8, 10, 12, 14, 24, 32, 36, and 48 h. The cell density and living cells were determined, respectively, by OD measurement at 600 nm and a plate count assay at 6 and 24 h.

Results

Detection of Differentially Expressed OM Proteins Using 2-DE-Based Proteomic Methodologies. Subproteomic methodologies were utilized for analysis of OM proteins of *E. coli* K12 K99+ separately cultured in buffered LBK medium of pH 6, 7.4, and 9.0. OM proteins were isolated using a sodium lauroyl sacrosinate (sarcosine) two-step method. SDS-PAGE showed that significant difference in band patterns was detected among the three cultures (data not shown). The OM proteins were analyzed using 2-DE. Figure 1 shows the micro-preparatively loaded (15 µg) 2-DE gels. The gels were stained with CBB R-250 and approximately 70 protein spots were detected (Figure 1a–c). The CBB R-250 stained 2-D gel protein patterns were quantitatively compared between pH 7.4 and 6.0 or 9.0 cultures. Protein spots showing 2-fold changes or above, either increase or decrease at expression level, were selected for further identification and functional characterization.

Out of the 70 protein spots, 11 proteins showed significantly differential expression between at least two different pH conditions were identified. The individual gels used to create the composites were subjected to pairwise comparison and quantitative analysis (Figure 1e–q). The 11 spots were identified as 9 uniquely proteins (Table 2). In addition, Tsx, FadL and OmpW showing over 1.5 fold change were also selected and characterized. All of the altered proteins were classified into five types of responses to pH as shown in Table 3: –) acid induced up-regulation at pH 6.0 as compared to pH 7.4 (OmpX and Dps); –) acid induced down-regulation at pH 6.0 as compared to pH7.4 (TolC and LambB); –) base induced up-regulation at pH 9.0 as compared to pH 7.4 (OstA, TolC, OmpT,

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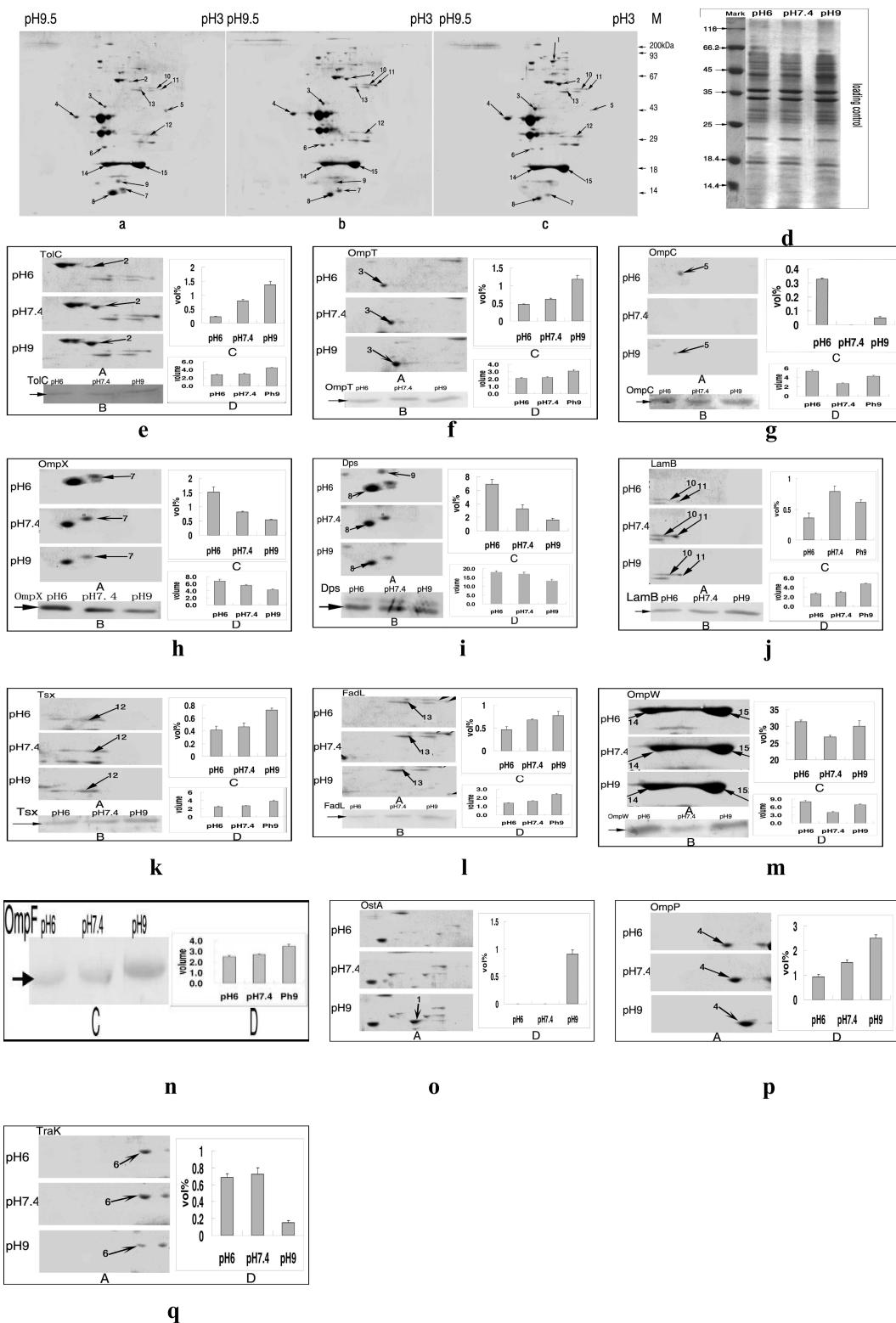


Figure 1. Subproteomic analysis of the altered OM proteins in response to pH. (a–c) 2-DE profile of OM proteins expressed in pH 6 (a), pH 7 (b), and pH 9 (c). Line M, molecular weight standards. Altered spots are shown by arrows. (d–o) A, Enlarged partial 2-DE gels showing the altered spots in 2-DE gels from cultures with pH 6, 7.4, or 9. C, Histogram displays besides showing the changes in spot intensity of the differential expressed proteins among the three pH concentrations. From left to right in each group, bars represent spot intensity obtained in pH 6, 7.4, and 9 concentrations. Vol (%) relative volume (according to Melanie 4.0 software description), volume divided by the total volume over the whole image. B, Immunoblot detection of altered spots. D, Histogram displays besides showing the changes in band intensity of the differential expressed proteins among the three pH concentrations. From left to right in each group, bars represent band intensity obtained in pH 6, 7.4, and 9. The relative volume is according to Phoretis 1D software description. d, SDS/PAGE analysis in the same gel as a loading control for the Western blotting. e, TolC; f, OmpT; g, OmpC; h, OmpX; i, Dps; j, LamB; k, Tsx; l, FadL; m, OmpW; n, OmpF; o, OstA; p, OmpP; q, TraK.

OM Proteins in Response to pH**research articles****Table 2.** Identification of *E. coli* K12 OM Proteins Shown in Figure 1 by PMF Searching and Subcellular Locations by Program PSORTb Version 2.0

spot no	protein description	report ^a	subcellular location	K-12 gene accession number	no. of peptides matched	M _r	score	vol %		
								pH 6	pH 7.4	pH 9
1	OstA		OM	ECK0055	15	89843	134	0	0	0.90 ± 0.08 ^c
2	TolC	P	OM	ECK3026	10	53967	87	^{b†} 0.22 ± 0.02	0.79 ± 0.07	1.58 ± 0.12
3	OmpT	G	OM	ECK0557	8	35540	83	0.47 ± 0.01	0.61 ± 0.03	^{b†} 1.28 ± 0.11
4	OmpP		OM	ECK0803	12	35477	146	0.93 ± 0.11 ^{b,b}	1.53 ± 0.09	3.11 ± 0.13 ^c
5	OmpC	G	OM	ECK2207	6	40343	73	0.32 ± 0.01 ^{b,b}	0	0.05 ± 0.01
6	TraK		OM		7	25610	119	0.68 ± 0.05	0.73 ± 0.07	0.15 ± 0.02 ^c
7	OmpX	PG	OM	ECK0803	6	16350	86	1.83 ± 0.17	0.82 ± 0.03	^{b†} 0.55 ± 0.01
8	Dps	P	unknown	ECK0801	8	17841	102	6.06 ± 0.57 ^c	2.68 ± 0.48	1.61 ± 0.27
9	Dps	P	unknown	ECK0801	7	17841	89	0.82 ± 0.12 ^c	0.59 ± 0.1	0
10	LamB	G	OM	ECK4028	8	47469	75	0.16 ± 0.03	0.29 ± 0.07	0.27 ± 0.02
11	LamB	G	OM	ECK4028	9	47469	77	0.20 ± 0.08	0.50 ± 0.09	0.34 ± 0.04
12	Tsx	G	OM	ECK0405	7	33568	63	0.40 ± 0.03	0.41 ± 0.06	0.72 ± 0.06
13	FadL	G	OM	ECK2338	8	48742	62	0.46 ± 0.07	0.68 ± 0.02	0.77 ± 0.09
14	OmpW		OM	ECK1250	5	25861	51	10.42 ± 0.22	6.43 ± 0.34	12.52 ± 0.92
15	OmpW	OM	ECK1250	3	22913	33	19.95 ± 0.33	13.42 ± 0.21	17.50 ± 0.78	

^a P: The result was obtained by 2-D analysis in a previous report. G: The result was obtained by gene analysis in a previous report. ^b Our result is different from that was reported, arrow refers to the tendency reported. ^c First reported here.

Table 3. Proteins Showing Differential Expression in Response to Different pHs

spot no.	protein	multiple to pH 7.4	ratio between pH 6 and 9
Acid-induced protein			
7	OmpX	2.23	3.23
8,9	DPS	2.1	4.27
Acid-repressed protein			
2	TolC	3.57	7.18
10,11	LamB	2.19	1.69
Base-induced protein			
1	OstA	appeared	appeared
2	TolC	2	7.18
3	OmpT	2.1	2.72
4	OmpP	2.03	3.34
12	Tsx	1.76	1.8
13	FadL	1.13	1.67
Base-repressed protein			
6	TraK	4.76	4.53
10,11	DPS	2.04	4.27
Acid- and base-induced protein, compared to pH 7.4			
5	OmpC	appeared	6.4
14,15	OmpW	1.51	1.01

OmpP, Tsx, and FadL); (4) base induced down-regulation at pH 9.0 as compared to pH 7.4 (TraK and Dps); (5) acid and base induced up-regulation at pH 6.0 and 9.0, but not at pH 7.4 (OmpC and OmpW). In addition, according to protein spot ratio between pH 6.0 and 9.0, these proteins could also be classified into the follow categories: (1) Expression decreased with pH from acid to base (OmpX and Dps); (2) Expression elevated with pH from acid to base (TolC, OstA, OmpT, OmpP, Tsx, and FadL); (3) Expression decreased in both acid and base conditions (LamB and TraK); (4) Expression elevated in both acid and base conditions (OmpC and OmpW) (Table 3).

Furthermore, Western blotting was utilized for the confirmation of protein expression changes using rabbit antisera against TolC, OmpT, OmpC, OmpX, Dps, LamB, Tsx, FadL, OmpW, or OmpF as the primary antibody and HRP-antirabbit IgG as the secondary antibody (Figure 1B). OmpF was not detected in our 2-D gel, but it was added as a target protein here since several

previous reports indicated that this porin and OmpC formed regulation pairs in response to salt, antibiotic and pH.^{29–32} Our data showed that the results obtained by Western blotting are coincident with those obtained by 2-DE except for Dps and LamB. For LamB, its expression was found to be up-regulated in base pH by Western blotting, while remained no change by 2-DE gel electrophoresis. For Dps, its expression was found to be down-regulated with increased pH by 2-DE gel electrophoresis, while remained no change among the three pH cultures by Western blotting.

Functional Characterization of These Altered OM Proteins Using Genetically Modified Strains with Gene Deletion. For functional characterization of the differential proteins in response to pH, genetically modified strains with the gene deletion of these proteins were utilized for analysis of pH-dependent growth. These strains included $\Delta tolC$, $\Delta ompT$, $\Delta ompC$, $\Delta ompX$, Δdps , $\Delta lamB$, Δtsx , $\Delta fadL$, $\Delta ompW$, and $\Delta ompF$, but not OstA, TraK and OmpP. The genetically modified strains were separately cultured in buffered LBK mediums with pH 4.5, 4.9, 6.0, 7.4, 9.0, 9.5 or 9.8. Figure 2a–g showed growth curves of these mutants and their parent strain *E. coli* K12 BW25113. There was no significant difference between the mutants and the control when they were cultured in mediums with pH 6, 7.4, or 9.0 (Figure 2c, d, e). However, significant differences in cell growth were detected at the extreme pH. In the medium with pH 4.9, the growth of $\Delta tolC$, $\Delta ompC$, Δdps , and $\Delta lamB$ was significantly different from that of the control (Figure 2b). Specifically, little growth was observed for $\Delta tolC$, and about 2/3 OD value of the control was detected for Δdps after 5 h of incubation. Very slow growth was detected during exponential phase (about 0.5 OD), and the recovery from the slow growth at 24 h was found for $\Delta ompC$ and $\Delta lamB$. Furthermore, almost all the mutants grew slower than control when they were cultured in medium with pH 4.5 (Figure 2a). $\Delta ompT$, Δtsx , $\Delta lamB$, $\Delta fadL$, and $\Delta ompW$, $\Delta ompF$ respectively, showed decreased growth of about 0.1 OD and 0.2 OD as compared to the control. $\Delta ompC$, $\Delta ompX$, and Δdps grew slower at pH 4.5 than at pH 4.9. Interestingly, no live bacteria were detected for $\Delta tolC$ at pH 4.5. Meanwhile, bacterial growth at base conditions was investigated. There was no significant

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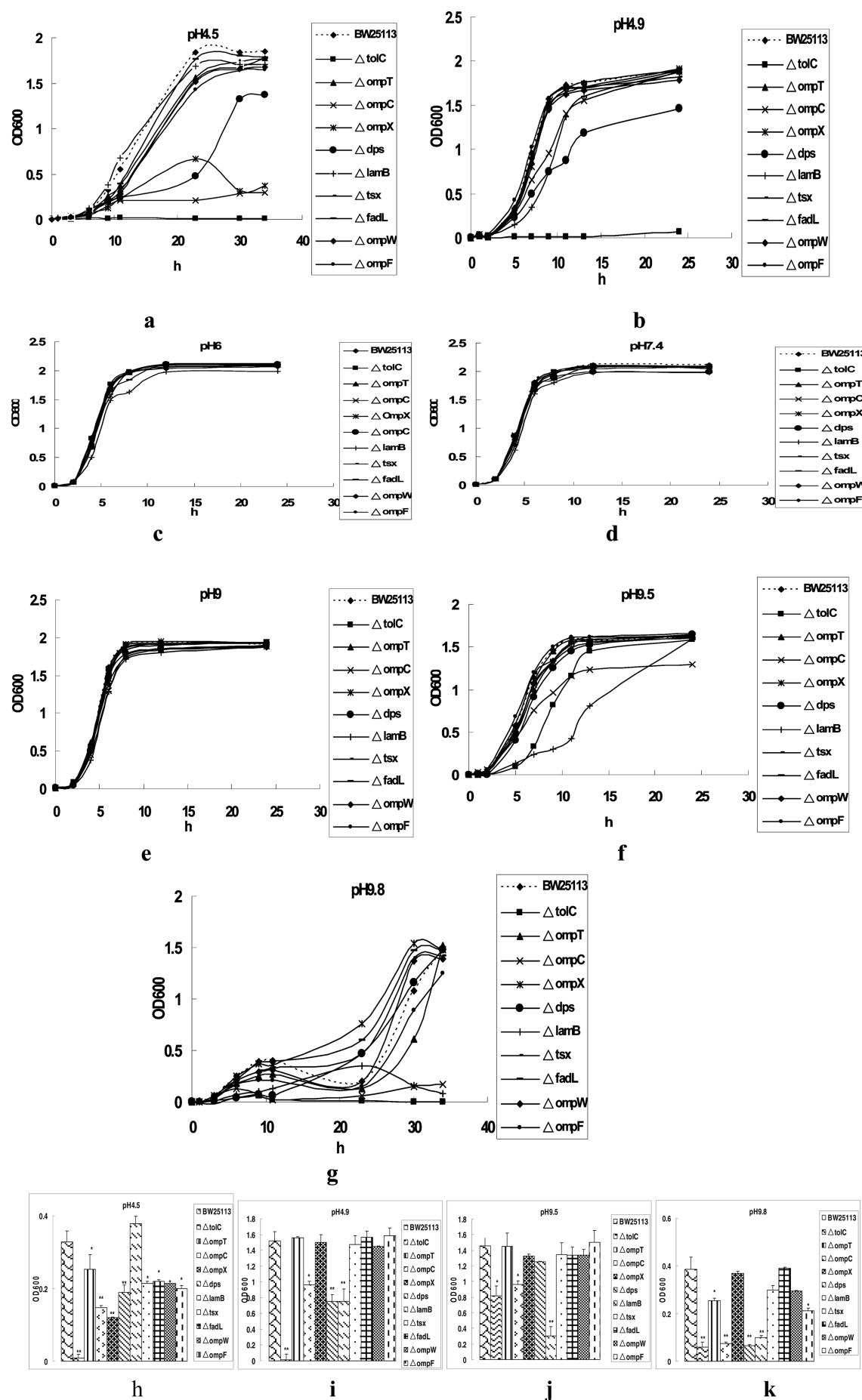


Figure 2
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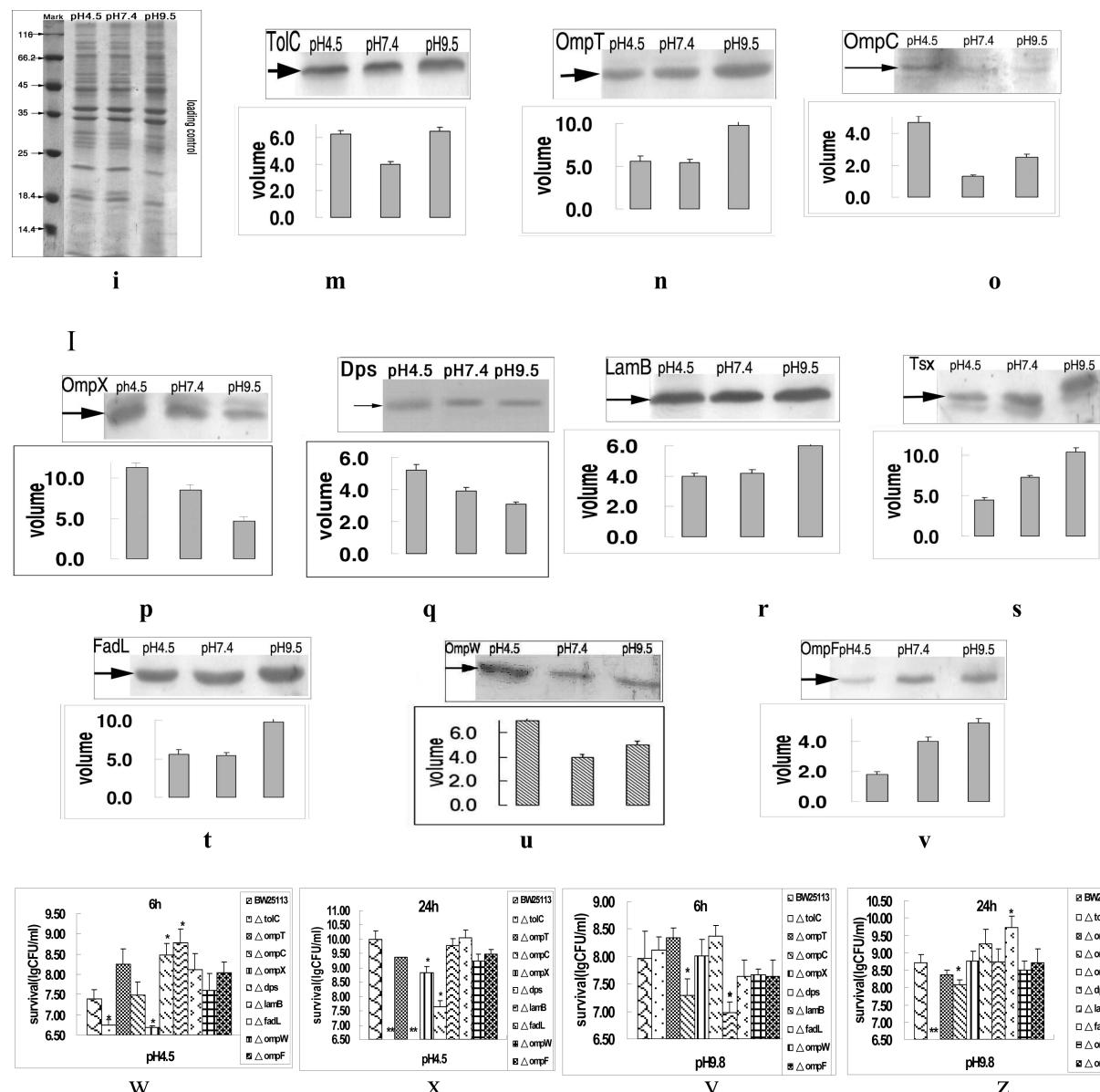


Figure 2. Functional characterization of genetically modified strains with gene deletion in response to the extreme pH. (a–g) Growth curve of *E. coli* BW25113 and its genetically modified strains with gene deletion of these altered OM proteins in medium with different pH. Error bars represent standard error of the mean. (a) pH 4.5; (b) pH 4.9; (c) pH 6; (d) pH 7.4; (e) pH 9; (f) pH 9.5; (g) pH 9.8. (h–k) Comparison in growth ability at 9 h. (l) SDS/PAGE analysis in the same gel as a loading control for the Western blotting. (m–v) Immunoblot detection of altered spots in response to pH 4.5, 7.4, and 9.5. (m) TolC; (n) OmpT; (o) OmpC; (p) OmpX; (q) Dps; (r) LamB; (s) Tsx; (t) FadL; (u) OmpW; (v) OmpF. (w–z) Comparison in survival ability of genetically modified strains in response to the extreme pH based on plate counting. Each bar shows the average of bacterial growth and standard deviation of three separate experiments for a–k and w–z. Double asterisks denote the datum points that differed very significantly ($P < 0.01$), and one asterisk denotes the datum points that differed significantly ($P < 0.05$) between mutants and their parent strain *E. coli* BW25113 by the independent-samples *t* test.

difference between the mutants and the control in medium with pH 9.5 except for $\Delta tolC$, $\Delta ompC$, and $\Delta lamB$ (Figure 2f). $\Delta ompC$ showed the same tendency with control, but its culture decreased about 0.3 OD. $\Delta tolC$ and $\Delta lamb$ lagged for about 1.1 and 0.6 OD at 11 h respectively, and then caught up with the growth of control at 24 h. When these bacteria were cultured in medium with pH 9.8, all of them including the control showed difficulty in survival during the first 24 h (Figure 2g). Later, $\Delta ompT$, Δdps , Δtsx , $\Delta fadL$, $\Delta ompW$, $\Delta ompF$, and the control partially recovered to about 0.5 to 1.5 OD at 30 h, whereas $\Delta lamB$ and $\Delta ompC$ showed the growth at lower than 0.3 OD. Like in the acidic condition, no live bacteria were

detected for $\Delta tolC$. Figures 2h–k are the comparisons among these genetically modified strains with gene deletion when they were cultured for 9 h. Compared with their parent strain, all mutants except for $\Delta lamB$ at pH 4.5, $\Delta ompT$, $\Delta ompX$, Δtsx , $\Delta fadL$, $\Delta ompW$, and $\Delta ompF$ at pH 4.9, $\Delta ompT$, $\Delta ompX$, Δdps , Δtsx , $\Delta fadL$, $\Delta ompW$, and $\Delta ompF$ at pH 9.5, and $\Delta ompX$, Δtsx , $\Delta fadL$, and $\Delta ompW$ at pH 9.8 grew significantly slower.

Western blotting was performed to investigate the expression of the altered proteins of *E. coli* K12 BW25113 at the extreme pH 4.5 and 9.5 (Figures 2m–v). It was found that TolC and OmpC were elevated at both extreme pH (Figure 2m, o), OmpX was elevated at pH 4.5 and decreased at pH 9.5 (Figure 2n).

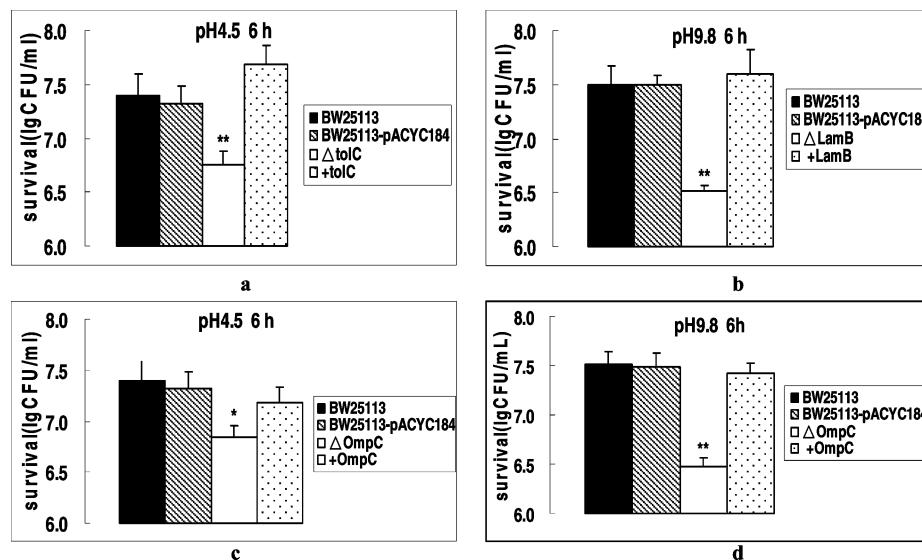


Figure 3. Functional characterizations of genetically modified strains with gene complementation in response to extreme pH. Each bar shows the average survival OD (600nm) and standard deviation of three separate experiments. Double asterisks denote the datum points that differed very significantly ($P < 0.01$), and one asterisk denotes the datum points that differed significantly ($P < 0.05$) between mutants and their parent strain *E. coli* BW25113 by the independent-samples *t* test. (a) Survival ability of $\Delta tolC$ complemented by *tolC* in pH4.5 at 6 h; (b) Survival ability of $\Delta lamB$ complemented by *lamB* in pH9.8 at 6 h; (c) Survival ability of $\Delta ompC$ complemented by *ompC* in pH4.5 at 6 h; (d) Survival ability of $\Delta ompC$ complemented by *ompC* in pH9.8 at 6 h.

and Tsx and OmpF was decreased at pH 4.5 or elevated at pH 9.5 (Figure 2s, v). In addition, Dps and OmpW were found to be elevated at extreme acidic conditions (Figures 2q, u), and OmpT, LamB, and FadL were elevated at extreme basic conditions (Figures 2n, r, t).

Furthermore, plate count assay was used to investigate the ability of the genetically modified strains with gene deletion to grow in medium with the extreme pH at 6 h or 24 h (Figures 2w–z). Compared to their controls, significant differences in growth rate were found in $\Delta ompX$ and $\Delta tolC$ (grew slower), and $\Delta ompT$, Δdps , and $\Delta lamb$ (grew faster) at pH 4.5 for 6 h (Figure 2w), in $\Delta tolC$, $\Delta ompC$, $\Delta ompX$, and Δdps (grew slower) at pH 4.5 for 24 h (Figure 2x), in $\Delta ompC$ and $\Delta lamb$ (grew slower) at pH 9.8 for 6 h (Figure 2y), and in $\Delta tolC$, $\Delta ompC$ (grew slower), $\Delta fadL$ (grew faster) at pH 9.8 for 24 h (Figure 2z). Very importantly, there was no growth of $\Delta tolC$ in both pH conditions. The results obtained by plate count were consistent with those obtained by OD measurement.

Functional Characterization of Key Altered OM Proteins Using Genetically Modified Strains and Gene Complementation Approach. The growth characterization of $\Delta tolC$, $\Delta ompC$, and $\Delta lamB$ at extreme pH above indicates the importance of these proteins in response to pH. Their complementation strains $+tolC$, $+ompC$, and $+lamB$ were constructed to identify the function of the deleted genes in this response (Figure 3). Our results demonstrated that $+tolC$ could survive at the extreme acid, $+lamB$ at the extreme base, and $+ompC$ at both the extreme acid and base. These results indicated that the survival abilities of the genetically modified strains were recovered in the four complemented strains, suggesting TolC, OmpC, and LamB play important roles in the pH-regulation.

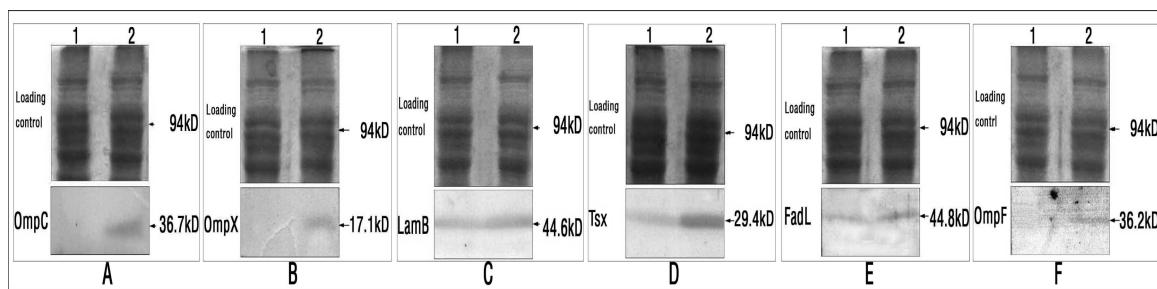
Investigation of Pathways of Altered OM Proteins of Host $\Delta tolC$ in Response to pH Change Using Death-Rescuing Approach. As described above, no cell growth was detected when $\Delta tolC$ was cultured at both extreme pH of acid and base conditions. Thus, we hypothesized that pathways that made $\Delta tolC$ survival under the extreme pH conditions should

be TolC-independent. Otherwise, they are TolC-dependent. In this regard, we developed a death-rescuing assay, in which $\Delta tolC$ was used as a host to harbor *ppompC*, *ppompX*, *pplamB*, *pptsx*, *ppfadL*, *ppompW*, or *ppompF*. The resulted strains were called $*ompC$, $*ompX$, $*lamB$, $*tsx$, $*fadL$, $*ompW$, and $*ompF$. These strains were cultured in media with the extreme pH and then induced by IPTG for induction of expression of these proteins (Figure 4a). As a result, $\Delta tolC$ could survive at the extreme acid of pH4.5 when it overexpressed OmpC, OmpX, LamB, FadL, or OmpW (Figure 4b), and at the extreme base of pH9.8 when it overexpressed LamB, FadL, OmpW, OmpF (Figure 4c). However, no cell growth was detected when the strain overexpressed Tsx, OmpF at pH 4.5 and OmpC, OmpX, Tsx at pH 9.8 (Figure 4d). These results indicate that the ability of TolC in response to pH could be recovered by elevated expression of OmpC, OmpX, LamB, OmpW, or FadL at the extreme acid condition, and LamB, FadL, OmpW or OmpF at the extreme base condition. Accordingly, the same pathway may be shared by TolC with Tsx and OmpF at pH 4.5, and with OmpC, OmpX, and Tsx at pH 9.8.

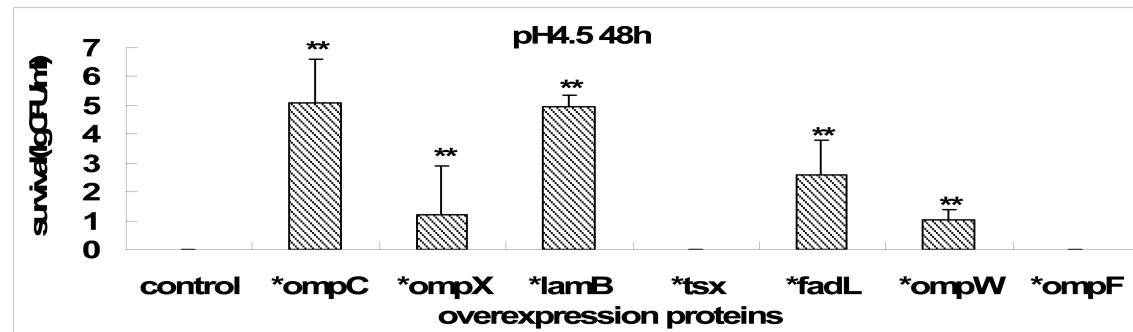
Profile of Altered OM Proteins in Response to pH. The altered OM proteins were ranked according to their magnitude changes at protein expression level, which could potentially function as an index of the importance of these proteins in *E. coli*'s response to extreme pH (Table 4). These results were generally consistent with those obtained from different functional assays. The three most important OM proteins, OmpC, TolC, and OmpX were the ones in response to acid, and TolC, OmpC, and LamB were the ones in response to base. These results indicate the importance of TolC, OmpC, LamB, and OmpX in the pH response of *E. coli*.

Moreover, we investigate association of the OM molecules with pH resistance at both protein and gene levels (Table 5).

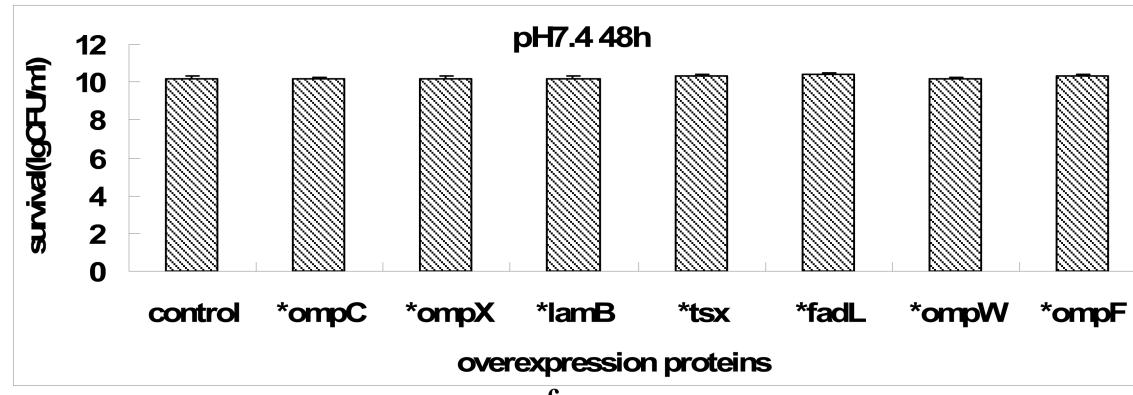
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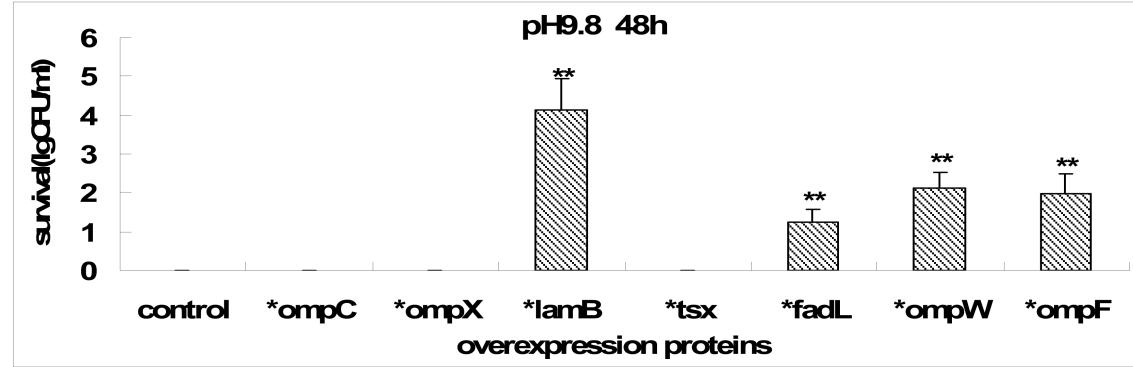
a



b



c



d

Figure 4. Functional characterization of Δ to/C harboring the gene of each of altered OM proteins in response to pH 4.5, 7.4 and 9.8 for 48 h based on bacterial count in Petri dish. Each bar shows the average of bacterial numbers and standard deviation of three separate experiments. Double asterisks denote the datum points that differed very significantly ($P < 0.01$), and one asterisk denotes the datum points that differed significantly ($P < 0.05$) between each of genetically modified strains and control Δ to/C (s) by the independent-samples t test. (a) Immunoblot detection of overexpressed OM proteins in Δ to/C with vector pLLP-ompA after induced by IPTG. 1, Δ to/C-pLLP-ompA; 2, Δ to/C harboring pLLP-ompA-omps; (b) pH 4.5; (c) pH 7.4, (d) pH 9.8.

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Table 4. Evaluation of Capabilities of the Altered OM Proteins in Response to pH

	acid					rank	base				
	2-DE	western blotting	bacterial growth	bacterial survival	total score		2-DE	western blotting	bacterial growth	bacterial survival	total score
TolC	2	5	1	1	9	2	3	4	1	1	9
OmpT	9	9	7	5	30	7	2	1	6	6	15
OmpC	1	1	3	1	6	1	1	3	2	2	8
Dps	5	7	4	3	19	4	4	6	6	6	22
LamB	4	9	8	7	28	6	5	2	3	3	13
FadL	8	8	9	8	33	8	8	9	6	5	28
Tsx	6	6					9	5			
OmpX	3	4	2	2	11	3	7	4	6	8	25
OmpW	7	3	6	4	20	5	6	8	5	4	23
OmpF	2	5	6				7	4	7		

Table 5. Summary of Functional Proteomic and Genomics Studies of *E. coli* in Response to Different pHs

protein	volume of western blotting						accordance of 2-DE with Western blotting	OD values of gene deletion mutants					numbers of live bacteria of gene deletion mutants		
	pH 6	pH 9	pH 6	pH 9	pH 4.5	pH 9.5		pH 4.5	pH 4.9	pH 9.5	pH 9.8	pH 4.5	pH 9.8	accordance of OD with live bacterial numbers	accordance of proteomics with gene deletion
TolC	↓	↑	↓	↑	↑	↑	OK	↓	↓	↓	↓	↓	↓	OK	OK
OmpT	—	↑	—	↑	—	↑	OK	↓	—	↓	—	—	—	OK	OK
OmpC	↑	↑	↑	↑	↑	↑	OK	↓	↓	↓	↓	↓	↓	OK	OK
OmpX	↑	↓	↑	↓	↑	↓	OK	↓	—	—	↓	—	—	OK	OK
Dps	↑	↓	—	↓	↑	↓	almost	↓	↓	—	↑	↓	↑	OK	OK*
LamB	↓	—	↓	↑	↓	↑	almost	↑	↓	↓	↑	—	↓	OK	OK*
Tsx	—	↑	—	↑	↓	↑	OK	—	—	—	—	—	—	OK	OK*
FadL	—	↑	—	↑	—	↑	OK	↓	—	—	—	—	—	almost	OK*
OmpW	↑	↑	—	↑	↑	↑	OK	—	—	↓	—	↓	↓	OK	OK
OmpF	—	↑	↓	↑	↑	↑	OK	↓	—	↓	↑	—	—	OK	OK

for Dps and OmpW in acid, and LamB in base. Isoform spots in 2-DE gels may somewhat contribute to the inconsistency. The accordance at the level of gene function is achieved except for *fadL* and *ompF* in acid. In the culture, $\Delta fadL$ and $\Delta ompF$ showed no change of survival contrasting lagged growth. At both protein and gene levels, these changes obtained from the two levels are in agreement with each other for TolC, OmpT, OmpC, OmpX, Dps, LamB and OmpW at both pH, and for Tsx and FadL at acid. Generally, the results obtained from functional characterization of the genetically modified strains are in consistence with those obtained from 2-DE-based proteomic studies.

Discussion

Differentially expressed proteins of bacteria in response to environmental stress are detected by differential proteomics. The bottleneck of the differential proteomics is to identify key target proteins involved in the response of bacteria to the changing environments.^{33,34} A line of evidence has indicated that not all of the differentially expressed proteins contribute to the biological processes of interests.^{35,36} Therefore, identification of key molecules among them is a question to be solved in current proteomics. In the present study, the altered proteins detected by proteomic methodologies were functionally validated by the investigation of growth of the genetically modified strains with the gene deletion of the altered OM proteins and the key pH regulating proteins were identified using complementation or overexpression approach. With the use of the approach, OstA, TolC, OmpT, OmpP, OmpC, Trak, OmpX, Dps, LamB, Tsx, FadL, OmpW, and OmpF were characterized as pH-related OM proteins. Out of these OM proteins, TolC, OmpC, OmpX, and LamB were found to play critical roles in the pH regulation.

Bacterial OM proteins work as groups in response to environmental stresses or changes. Previous reports indicate that the expression of OmpC and OmpF is regulated by EnvZ-OmpR or CpxA-CpxR components which belong to two distinct two-component systems, but the function and regulation network of OM proteins are largely unknown. It has been postulated that they may play multiple roles and be involved in multiple pathways during bacterial response to environmental changes. In the present study, we identified some of OM proteins that are involved in pH response of *E. coli* including TolC. $\Delta tolC$ could not survive at the extreme pH. We hypothesized that the proteins shared the same pathway with TolC could not make $\Delta tolC$ alive at the extreme conditions when they overexpressed in $\Delta tolC$ deleted strain. On the contrary, the proteins that kept $\Delta tolC$ alive under extremely pH conditions should belong to TolC-independent pathways. To test this hypothesis, a death-rescuing assay was developed in our laboratory to determine which OM proteins were involved in TolC-dependent or -independent pathways. Our results obtained from the protein overexpression study using $\Delta tolC$ indicate that OmpC, OmpX, LamB, FadL, OmpW, and LamB, FadL, OmpW, OmpF respectively function in TolC-independent pathway at the extreme acid and extreme base. TolC may share the same pathway with OmpF and Tsx at the extreme acid, and with OmpX, OmpC, and Tsx at the extreme base. These complementary proteins contribute to survival of TolC-deleted bacteria cultured under the extreme pH conditions. Our recent evidence has uncovered the network of OM proteins in response to streptomycin. Taking all these results together, an analysis of the OM network and its regulation provides better understanding of mechanisms of bacteria in response to environmental stresses or changes.

OM Proteins in Response to pH

Out of these altered proteins, TolC and Dps have been reported to be altered at protein expression level,^{38–40} and *ompC*, *ompT*, *lamb*, *fadL* and *tsx* at gene expression level,⁴¹ and OmpX (*ompX*) at both protein and gene expression levels.¹⁹ Thus, our results revealed for the first time the role of OmpC, Dps and OmpP at acid, and OstA, OmpP, TraK at base. Meanwhile, our results demonstrated the role of OmpC, OmpT, LamB, FadL and Tsx at protein expression level. On the other hand, there were some different results between the present study and other reports. These differences from the other reports included (1) TolC was acid-induced³⁸ rather than acid-repressed; (2) OmpT was base-repressed⁴¹ rather than base-induced; (3) OmpX was both acid- and base-induced¹⁹ rather than only acid-induced. These differences may be related to culture conditions or assays used. Stancik et al. showed an acid- and base-induced OmpX relative to pH 7¹⁹ and Yohannes et al. reported acid-induced TolC under anaerobiosis,³⁸ whereas our analysis was from the growth cultured under aerobiosis. OmpT was reported to be base-repressed using gene array hybridization assay,⁴¹ which may be different from the result obtained from proteomics approach. It is well-known that results from microarray studies are inconsistent with those from 2-D gels under some circumstance, and microarray and proteomics detections reflect the changes of gene and protein expression levels, respectively.^{43,44}

In summary, a functional OM proteome in response to pH was investigated and determined by both proteomic and genetic approaches. Out of the OM proteome, TolC, OmpC, LamB and OmpX were identified as key OM proteins involved in pH response of *E. coli*. OstA, TraK, and OmpP were first reported to be involved in pH regulation, and OmpC and Dps to be induced in acid condition. In addition, utilizing death-rescuing assay developed in our laboratory, we identified TolC-dependent and -independent pathways mediated by the altered OM proteins and the potentially functional OM network for pH-regulation in *E. coli*.

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