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Identification of outer membrane proteins of *Edwardsiella tarda* in response to high concentration of copper

Chao Wang, Haili Zhang, Hui Li, Xuan-xian Peng

Center for Proteomics, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, People's Republic of China

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Abstract

The antimicrobial properties of copper have been reported, but little is known about the outer membrane proteins regulating copper resistance. In the present study, a sub-proteomic approach was utilized to investigate altered outer membrane (OM) proteins of *Edwardsiella tarda* in response to $CuSO_4$. Upregulation of HemR and dwonregulation of Imp, EvpB, TolC, ETAE_2935, ETAE_1480, and ETAE_1723 were detected in *E. tarda* EIB202 survived in 1.0 mM $CuSO_4$ compared with the control without the ion. These alterations were validated, at random, using Western blotting. They were first revealed here to be bacterial copper-resistant proteins in combination of protein homology analysis. These findings highlight the way to clarify copper-resistant mechanisms.

Keywords: Copper resistance; Outer membrane proteins; *E. tarda*; Proteomics.

1. Introduction

Copper contributes to the function of numerous essential metabolic processes and thus is an essential micronutrient element required by almost living organisms [1]. However, copper at increased levels is also a unique metal known for its antimicrobial properties. The mechanism of copper ions toxic to cells has been extensively studied [2-4]. The most important antimicrobial mechanisms include:1) Elevation of oxidative stress inside a cell result in oxidative damage to cells; 2) Decline in the membrane integrity of microbes leads to leakage of specific essential cell nutrients and subsequent cell death; 3) Unsuitable binding of copper to proteins that do not require copper for their function causes loss-of-function of the protein, and/or breakdown of the protein into nonfunctional portions.

The antimicrobial properties of copper are still under active investigation due to incompleteness of our knowledge to antimicrobial role of copper. Recently, high throughput proteomics approach has been used to investigate the antimicrobial role of copper in *Escherichia coli, Edwardsiella tarda, Pseudomonas fluorescens*, *Pseudomonas putida*, *Lactococcus lactis* [5-9]. These studies elucidate the differential protein expression of these bacteria when exposed to copper stress, and indicate that the proteins from copper stressed cells exhibited a higher degree of oxidative proline and threonine modifications. However, information on alteration of OM proteins in response to copper stress is still absent.

Edwardsiella tarda is an uncommon enteric bacterium which has been found generally in animal hosts and occasionally in human feces [10]. Edwardsiellosis caused by the bacterium has been implicated in gastroenteritis, meningitis, biliary tract infections, peritonitis, liver and intra-abdominal abscesses, wound infections and septicaemia [11, 12]. The

***Corresponding author:** Dr. Xuanxian Peng, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, University City, Guangzhou 510006, People's Republic of China. Phone Number: +86-20-3145-2846; Fax Number: +86-20-8403-6215; E-mail Address: pxuanx@sysu.edu.cn; wangpeng@xmu.edu.cn

disease has caused severe economic losses in the fish farming industry worldwide. However, how to control the disease is still under investigation. A recent report has indicated that prolonged exposure to copper has multiple effects on *E. tarda* TX5 and results in significant attenuation of bacterial virulence [13]. In the present study, two-dimensional gel electrophoresis (2-DE) based proteomics was used to detect OM proteins of *E. tarda* EIB202 survived in medium with high concentration of $CuSO_4$. Seven unique proteins were identified. Out of them, six were downregulated and one was upregulated. These results may have significant implications in an understanding of mechanisms of *E. tarda* survived in high concentration of copper.

2. Material and Methods

2.1 *Bacterial strain and culture*

E.tarda EIB202, which complete genome sequence has been disclosed in 2009 [14], was kindly provided by Professor YX Zhang, East China University of Science and Technology, China. The bacterium was grown at 30°C in tryptic soy broth (TSB) medium overnight, and then diluted in 1:100 separately using the medium with or without 1.0 mM $CuSO_4$. The cultures were harvested at an OD_{600} of 1.0 by centrifugation at 4,000g for 15min at 4°C.

2.2 *Extraction of OM membrane proteins*

The harvested bacterial cells were used for isolation of OM membrane proteins according to a procedure described previously [15]. Briefly, the cells were washed in sterile saline for three times, and then resuspended in sterile saline. Cells were disrupted by intermittent ultrasonic treatment. Unbroken cells and cellular debris were removed by centrifugation at 5,000g for 20 min and the supernatants were collected and were further centrifuged at 100,000g for 40 min at 4°C in a Beckman Coulter L-100XP centrifuge using a SW 41Ti Rotor. The precipitation was dissolved by 2% (W/V) sodium lauryl sarcosinate at room temperature for 30 min and ultracentrifuged again. The collecting pellets were resuspended in 50mM Tris-Cl and stored at -80°C. Concentrations of these proteins in the final preparation were determined using the Bradford method.

2.3 *2-DE and MALDI-TOF analysis*

2-DE was performed according to a procedure described previously [15]. Briefly, OM protein extracts containing 200 μg of proteins were dissolved in lysis solution and then rehydrate a pH 3-10 linear IPG strip (11 cm length, Bio-Rad). The total focusing was to be 40,000 Vhr and the maximum voltage was 8,000V using the Multiphor II system (Amersham). After the equalized by 2% (W/V) DTT and 2.5% (W/V) IAA (4M urea, 20% glycerol, 10% SDS) for 15 min, respectively, the IPG strips were transferred to the second-dimension electrophoresis using 12% acrylamide gels and stained with Coomassie Blue-R250. 2-DE gels were scanned in ImageScan and analyzed with ImageMaster 5.0 software (Amersham Bioscineces, Sweden). Altered spots were standardized and then compared based on their volume percentages in the total spot volume over the whole gel image. Significantly changed spots were selected by rate increased / decreased ≥ 2-fold or complete appearance and disappearance. Protein spots of interest were cut from the gels for analysis using Matrix-Assisted Laser Desorp-tion/ Ionization-Time of Flight Mass Spectrometry (MALDI-TOF). All MALDI analysis was performed by a fuzzy logic feedback control system (Reflex III MALDI-TOF system, Bruker) equipped with delayed ion extraction. Peptide mass finger printings were searched using the program Mascot (Matrix Science, London, U.K.) against the NCBI database; *E. coli* species database was defined as a matching species; one missed cleavage per peptide was allowed and the mass tolerance was 100 ppm. The protein subcellular locations were determined by Program PSORTb version 2.0 (http://www.psort.org/psortb).

2.4 *Western blotting*

Western blotting was performed as previously described [15]. Mouse antisera to TolC or EvpB were obtained from Fuji Bio Sci Tech Corp (Ji'an, China). Briefly, 1-DE and 2-DE gels were transferred to NC membranes for 3 h at 100 mA in transfer buffer (48 mM Tris, 39 mM glycine and 20% methanol) at 4 °C. The membranes were blocked with 5% skim milk and then incubated with mouse antisera to TolC or EvpB as the primary antibodies. The horseradish peroxidase (HRP) conjugated goat anti-mouse antibody was used as the secondary one. Antibody-tagged protein spots were detected by DAB.

2.5 *Phylogenetic analysis*

A BLAST search was performed on the sequences of these altered proteins using NCBI database as described previously [16]. A multiple sequence alignment was created with the amino acid sequences of YP_003294661.1 (ETAE_0603, Imp), YP_003296476.1 (ETAE_2430, EvpB), YP_003294249.1 (ETAE_0191, TolC), YP_003296979.1 (ETAE_2935), YP_003295532.1 (ETAE_1480), YP_003295773.1 (ETAE_1723), YP_003295845.1 (ETAE_1797, HemR) using ClustalX. Unrooted phylogenetic tree was constructed based on the sequences above using MEGA version 4.0 (http://www.megasoftware.net), and then was adjusted by TreeView to make it more readable. The sequences used in the trees were showed in Supplemental Figure 1. Bootstrap tests at 1000 replicates were carried out to examine the validity of the branching topologies. The numerical value of branch node reflects the confidence level. Values > 70 indicate statistical probability, while values < 50

are not statistically significant.

3. Results

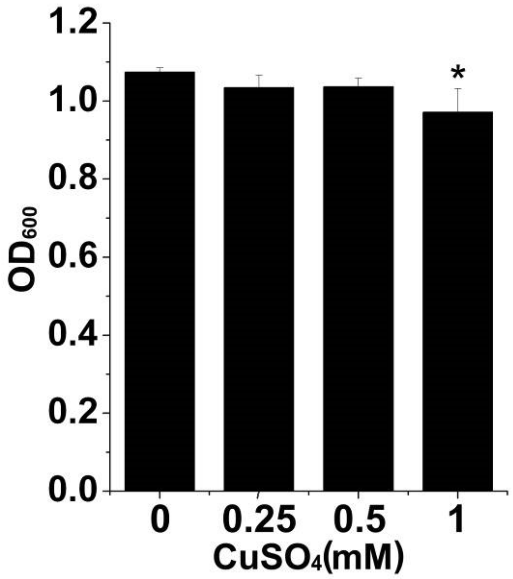

Figure 1. OD value of EIB202 cultured in medium with different concentrations of $CuSO_4$

3.1 *Effect of $CuSO_4$ on EIB202 growth*

EIB202 cells were cultured in TBS medium with 0, 0.25, 0.5 and 1.0 mM of $CuSO_4$. These cultures were harvested when the bacterial cell density in medium without the ions arrived at approximately 1.0 OD. Significantly inhibitory growth was found in bacteria cultured in medium with 1.0 mM $CuSO_4$ (Figure 1).

3.2 *Identification of differential OM proteins of EIB202 in response to $CuSO_4$*

To investigate an altered OM proteome of *E. tarda* in response to copper (II) ions, a sub-proteomic approach was utilized to identify differentially expressed proteins of sarcosine-insoluble fractions from EIB202 survived in medium with 1.0 mM of $CuSO_4$ (Cu-S). Approximately 40 protein spots were visualized in each of the gels stained with CBB R-250 (Figure 2A, B). Out of them, eight from Cu-S showed significant changes at the level of protein expression. They were named as 1 - 8. Figure 2C was an expanded view of the spots in the gels and Figure 2D showed the mean and standard deviation of these spots. They were identified as seven uniquely proteins. Table 1 showed the identities of these spots which were obtained by mass spectrometric analysis.

Table 1. Identification of Altered Spots by PMF or peptide fingerprinting Searching

Spot No.	Accession name	NCBI accession No.	Locus_tag	Protein description	Subcellular location	No. of peptides matched	No. of peptides unmatched	No. of peptides searched	Cover %	Mr/pI	NCBI score
1	D0ZC49_EDWTE	gi|269137961	ETAE_0603	organic solvent tolerance protein (Imp)	M/OM	50	40	90	52	90359/5.45	422
2	D0ZC49_EDWTE	gi|269137961	ETAE_0603	organic solvent tolerance protein (Imp)	M/OM	53	54	107	58	90359/5.45	435
3	D0ZB31_EDWTE	gi|269139775	ETAE_2430	type VI secretion system protein (EvpB)	unknown	33	54	87	57	54499/5.16	302
4	D0Z9U8_EDWTE	gi|269137549	ETAE_0191	outer membrane channel protein (TolC)	OM	17	21	38	49	51409/6.64	183
5	D0ZDY9_EDWTE	gi|269140278	ETAE_2935	nucleoside-specific channel-forming protein (Tsx)	OM	11	22	33	32	32786/5.62	116
6	D0ZH45_EDWTE	gi|269138831	ETAE_1480	MltA-interacting MipA family protein	unknown	17	74	91	39	31771/7.10	148
7	D0Z7Z3_EDWTE	gi|269139072	ETAE_1723	hypothetical protein ETAE_1723	unknown	8	54	62	47	12695/7.67	112
8	D0Z865_EDWTE	gi|269139144	ETAE_1797	hemin receptor precursor (HemR)	OM/M	35	55	90	58	72809/6.29	302

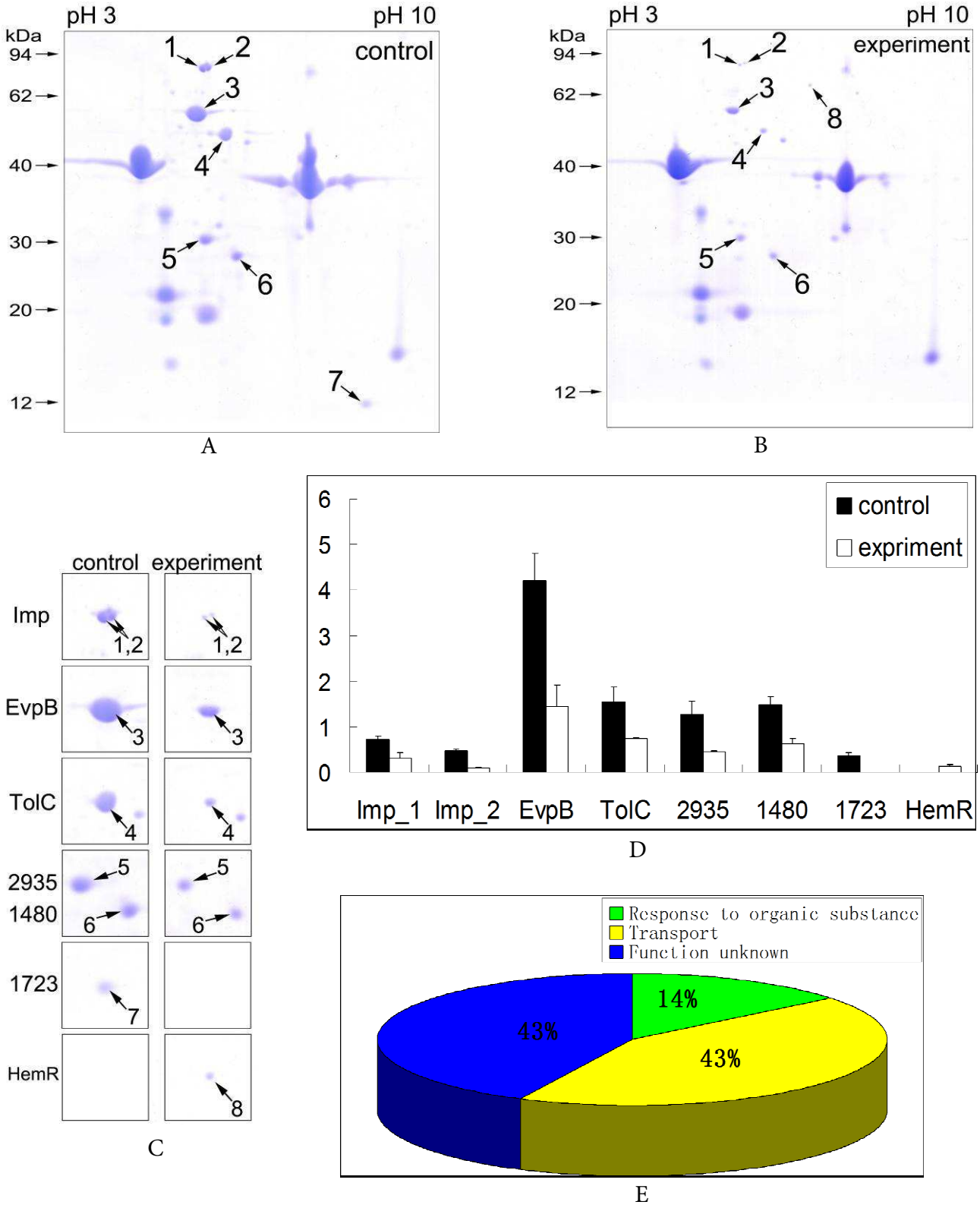

Figure 2. 2-DE subproteomics for investigation of altered OM proteins of *E. tarda* in response to copper. A, A representative 2-DE map of the sarcosine-insoluble fraction of Cu-C grown in TBS medium. B, A representative 2-DE map of the sarcosine-insoluble fraction of Cu-C grown in TBS medium. C, Enlarged partial 2-DE gels showing altered expression spots. D, Histogram displaying the changes in spot intensity of altered proteins between F-plasma-S-O (black) and F-plasma-S (white). Bars represent spot intensity, with the relative volume divided by the total volume over the whole image, according to ImageMaster version 5.0. E, Distribution of altered proteins based on GOA classification.

They were Imp (spot 1 and 2), EvpB (spot 3), TolC (spot 4), ETAE_2935 (spot 5), ETAE_1480 (spot 6), ETAE _1723 (spot 7), HemR (spot 8). Out of them, HemR was appeared and the others were decreased in Cu-S with respect to Cu-C. Except for EvpB, ETAE_1480 and ETAE_1723, which were proteins of unknown location, the other four were OM proteins. They are classified as response to organic substance (Imp), transport (TolC, ETAE_2935, HemR), function unknown (EvpB, ETAE_1480, ETAE_1723) (Figure 2E).

3.3 *Validation of differential proteins using Western blotting analysis*

To confirm these altered OM spots obtained from the 2-DE based proteomics, we randomly compare the difference of TolC and EvpB expression between Cu-C and Cu-S with 0.5, 1.0 mM concentrations of $CuSO_4$ using Western blotting. The Western blotting results indicated that downregulation of the two proteins was detected with the elevated concentrations of $CuSO_4$ (Figure 3). There was a better connection in TolC and EvpB between 2-DE and Western blotting. These results not only further demonstrated that TolC and EvpB were Cu-regulated expression proteins, but also indicated that there was a high confidence for the 2-DE based proteomics.

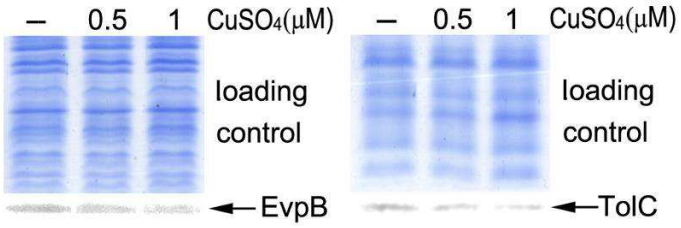

Figure 3. Confirmation of TolC and EvpB related to copper

3.4 *Phylogenic tree construction of altered proteins*

Since information regarding to role of the altered proteins from *E. tarda* is largely absent, homology analysis was carried out to find the homology proteins from other bacteria for the functional analysis. Based on the amino acid sequences of the seven proteins from EIB202 deposited in GenBank, BLAST homology searching yielded the following closest matches. The unrooted phylogenetic analysis showed a close genetic relationship of the full-length proteins in EIB202 with more than ten Gram-negative bacteria including *Escherichia coli* (Suppl Figure 1). These results indicate that high homology between these EIB202 proteins and the proteins from other bacteria. We may understand role of the altered proteins in combination of the information of their homologies.

4. Discussion

In the present study, the OM proteome of *E. tarda* EIB202 in response to copper was identified. They were Imp, EvpB, TolC, ETAE_2935, ETAE_1480, and ETAE_1723. They belong to different functional classes of response to organic substance (Imp), transport (TolC, ETAE_2935, HemR), function unknown (EvpB, ETAE_1480, ETAE_1723) according to the EMBL-EBI GOA database (www.ebi.ac.uk/GOA). These proteins are first reported here to be involved in copper resistance.

Since the role of *E. tarda* proteins is largely unknown, we constructed the phylogenic tree of these proteins from genetically close bacteria for understanding whether their homologies showed the role. Imp is one of organic solvent tolerance proteins that work for level of tolerance to organic solvents and the transport of lipopolysaccharide [17]. EvpB is one of type VI secretion system proteins that are widely distributed in pathogenic Gram-negative bacterial species [10]. TolC functions as a channel of the AcrAB-TolC multidrug efflux system. A report indicated that BaeSR, AcrD and MdtABC contribute to copper and zinc resistance in Salmonella [18]. Tsx is known to be involved in the permeation of nucleosides across the outer membrane under limiting substrate conditions and iron transport [19, 20]. MipS is an MltA-interacting MipA family protein. *E. coli* MipA serves as a scaffold protein required for the formation of a complex with MrcB/PonB and MltA and has recently been found to be involved in glucose regulation [19]. This complex could play a role in enlargement and septation of the murein sacculus and ETAE_1723 is a hypothetical protein. ETAE_1797 is a hemin receptor precursor. The receptor is involved in the utilization of heme and its protein complexes as iron sources in some of pathogenic bacteria [20]. In summary, TolC and hemin receptor are indirectly related copper metabolism, whereas the other five are first reported here to be copper-responsive proteins.

5. Concluding Remarks

In summary, a copper-responsive OM proteome is revealed in the present study. Out of the seven altered proteins, six were downregulated and four worked for transport. These results provide the proof on decline in the membrane integrity of microbes in response to high concentration of copper, but also show which OM proteins play the role in copper resistance.

6. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/91/0

Supplementary material includes Figure 1. showing the unrooted phylogenetic analysis

Acknowledgements

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