Immunoproteomics of membrane proteins of *Shigella flexneri* 2a 2457T

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Ying TY, Wang JJ, Wang HL, Feng EL, Wei KH, Huang LY, Huang PT, Huang CF. Immunoproteomics of membrane proteins of *Shigella flexneri* 2a 2457T. *World J Gastroenterol* 2005; 11(43): 6880-6883 http://www.wjgnet.com/1007-9327/11/6880.asp

INTRODUCTION

The genus *Shigella* spp. is a group of Gram-negative enteric bacilli which cause bacillary dysentery in human beings, accounting for 20% of the 4.6 million diarrhea-associated deaths among children[1]. Though the LPS can induce a good immune response in human beings, the role of proteins (especially the membrane proteins) in conferring immunity to shigellosis is at best speculative. Considering outer membrane proteins of *Shigella* spp. function as a dynamic interface between the cell and its surroundings, it is possible to develop new antigens from them. Due to the methodology limitations of protein separation and identification, it is difficult to identify the immunogenic proteins in bands on 1-D gel. With the improvement of 2-DE in recent years much valuable information is available and immunoproteomics has been built around 2-DE and routine immunologic technologies.

*S. flexneri* 2a is the dominant serotype causing shigellosis in China. Our laboratory has finished a two-dimensional electrophoresis reference map and a proteomic database of *S. flexneri* 2a 2457T[2], but only a few of membrane proteins can be identified in that database. In order to develop new protective antigens against *S. flexneri* and to understand their immune mechanism, we applied immunoproteomic technologies in screening new antigens of *S. flexneri* 2a 2457T.

MATERIALS AND METHODS

**Bacterial strains and growth conditions**

*S. flexneri* 2a 2457T was aerobically cultured in LB overnight at 37 °C. Overnight cultures were diluted 1:100 and shaken at 250 r/min. Growth was stopped at the early stationary phase at an *A*<sub>600</sub> of 3.3.

**Membrane protein preparation**

Cells were harvested and centrifuged for 15 min at 2 000 r/min (Sigma 3K12, No. 12150; St. Louis, MO, USA) at 4 °C. The pellet was washed thrice for 10 min at 2 000 r/min with low...
salt washing buffer (3 mmol/L KCl, 1.5 mmol/L KH₂PO₄, 68 mmol/L NaCl, 9 mmol/L NaH₂PO₄) [9]. Proteins were extracted using the ReadyPrep™ protein extraction kit (Membrane I) (BioRad, USA). Integral membrane proteins were separated from hydrophilic proteins using the nonionic detergent Triton X-114.

**Two-dimensional electrophoresis**

Eighteen-centimeter immobilized pH gradient (IPG) strips (pH ranges, 4-7) (Amersham Pharmacia Biotech, Sweden) were used. Isoelectric focusing (IEF) was conducted for 60 000 Vh (IPGphor, Amersham Pharmacia Biotech). Vertical slab SDS-PAGE (12.5%) was run at 30 mA/gel for the second dimension. Gels were stained with Colloidal Coomassie Blue [10]. Image analysis was performed with Image-Master 2D Elite Version 3.1.

**Preparation of antisera against 2457T**

*S. flexneri* 2a 2457T was aerobically cultured in LB overnight at 37 °C. Rabbits were immunized six times with culture solution intravenously at intervals of 5 d. The doses were (5, 7.5, 10, 15, 20, 20)×10⁸ CFU, respectively. Eight days after the last immunization, blood was collected from the tested animals and the sera were separated. Antibody titers 1:5 120 was measured by microagglutination test and ELISA.

**Immunoblot assay**

After two-dimensional electrophoresis, the gels were electroblotted onto Hybond™ECL™ nitrocellulose membrane (Amersham Pharmacia Biotech) using a semi-dry transfer unit (Hofer™ TE 77, Amersham Pharmacia Biotech, Sweden). Before immunodetection, the membranes were stained for 10 min with 5 g/L Ponceau S in 10 mL/L acetic acid and the positions of some selected spots were marked by clean needles. Western blotting was performed as previously described [5]. Then antigen-antibody complexes were detected with peroxidase-labeled goat anti-rabbit IgGs and substrate.

**In-gel protein digestion and MALDI-TOF-MS protein identification**

In-gel protein digestion was performed as previously described [5]. All MALDI-MS measurements were performed on a Bruker Reflex. III MALDI-TOF-MS (Bruker Daltonik, Bremen, Germany) operating in reflectron mode.

**Nanospray ESI-MS/MS**

The peptide solution after in-gel protein digestion was desalted with ZipTip C₁₈™ pipette tips (Millipore, Bedford, MA, USA). Electrospray ionization (ESI-MS/MS) was carried out with a hybrid quadrupole orthogonal acceleration tandem mass spectrometer (Q-TOF2) (Micromass, Manchester, UK) [9].

**Peptide mass fingerprinting**

Peptide mass fingerprinting searches were performed using the program Mascot developed by Matrix Science Ltd (http://www.matrixscience.com). For protein identification, peptide mass searches against the database of 2457T by Mascot licensed in-house and the searches against the NCBInr database with free access on the internet were done. A peptide mass accuracy of 0.3 Da was defined.

**RESULTS**

The sample was prepared on the basis of the separation of membrane proteins by temperature-dependent phase partitioning using Triton X-114 detergent. Proteins anchored to the membrane or containing one or two transmembrane domains were efficiently partitioned to the detergent-rich phase. In order to solubilize the protein thoroughly, thiourea was used. In pH 4-7 gradient 2-DE map, 148 spots were cut and 111 spots were successfully identified by MALDI-TOF-MS presenting 82 protein entries. Twenty-five proteins were not observed/identified in our previous work [3]. The majority of these 25 proteins (data not shown) were hydrophobic and associated with the membrane. The relative abundance of membrane-associated proteins identified in this study was higher than that in our previous study [3].

On the basis of the established immunoproteomic map of soluble proteins of *S. flexneri* 2a 2457T (unpublished), we described a group of spots in a 2-DE map of immunogenic proteins from hydrophobic proteins in this study. Five hundred micrograms of protein sample was used to perform the 2-DE. One of the parallel gels was electroblotted onto nitrocellulose membrane and the other was stained with Coomassie brilliant blue G-250. We successfully identified 20 immunoreactive spots from Coomassie brilliant blue stained gels using sera from immunized rabbits, which represented 13 protein entries, 5 known antigens and 8 novel antigens. The 20 spots were marked on the 2-D gel and corresponding blotting membrane (Figure 1). Table 1 lists all the identified proteins. ESI-MS/MS was used to confirm the protein marked as spot 1. Figure 2 shows the result of ESI-MS/MS identification.
Acquisition and archiving of information for gi|30064737 Pgm
Guyonnet F, Chastel C. Polypeptide Cell envelope Outer membrane protein 3a (II*; G; d)
Lipoamide dehydrogenase (NADH) Tsf
Phosphoglucomutase can be significantly YaeT
Gluconate-6-phosphate dehydrogenase Cell envelope
Cellular role Hypothetical protein
Galactose-binding transport protein; receptor for galactose taxis DnaK
D15
Cell envelope
TufB
Gnd
L S
Energy metabolism MopA
Heat shock protein Protein fate
Chaperone Hsp70; autoregulated heat shock protein S
Gene symbol gi|30063593 GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein Proportion
Protein synthesis
OppA
yMax
Protein common name Protein fate
OmpA
Energy metabolism
Transport and binding proteins gi|30061734 Protein chain elongation factor EF-Tu
Protein chain elongation factor EF-Ts
OmpA, a major and highly conserved outer membrane protein 3a (II*; G; d) is a precursor of
our other works [7-11]. The outer membrane protein 3a (II*; G; d) is a precursor of OmpA, a major and highly conserved outer membrane protein of Gram-negative bacteria. Due to its high copies per cell[12], multiple charged isoforms[13] and its strong immunogenicity, identification of OmpA was performed several times during the immunoproteomics analysis. All these proteins were observed in our other works (unpublished).

Novel antigens
Besides the above confirmatory findings, the study detected several new immunoreactive proteins (AtpA, OppA, MglB, LpdA, ClpB, Gnd, Pgm, YaeT). AtpA, LpdA, Gnd, and Pgm are components of the energy metabolism system. ATP synthesis/hydrolysis occurs in the ATP synthase F1 sector which lies at the surface of cytoplasmic membrane. LpdA codes for an outer membrane lipoamide dehydrogenase that is highly immunogenic. It is an in vivo-induced antigen in Mycobacterium tuberculosis[16]. Since LpdA is a functional subunit of both pyruvate dehydrogenase (aceEF) and alpha-ketoglutarate dehydrogenase (sucAB), a lpdA mutant of H. influenzae can be significantly attenuated[15]. Gnd is an important component of pentose phosphate pathway. Phosphoglucomutase (pgm) is associated with virulence of Brucella abortus because the deltapgm strain is unable to assemble the O side chain in the complete LPS. Vaccination with the deltapgm strain induces effective protection[16].

Figure 2 Mass spectra showing the determination of a partial peptide sequence of the hypothetical protein (spot 1).

DISCUSSION

Known antigens
Our results are in accordance with other studies[7-11]. The outer membrane protein 3a (II*; G; d) is a precursor of OmpA, a major and highly conserved outer membrane protein of Gram-negative bacteria. Due to its high copies per cell[12], multiple charged isoforms[13] and its strong immunogenicity, identification of OmpA was performed several times during the immunoproteomics analysis. All these proteins were observed in our other works (unpublished).

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Protein synthesis
OppA
yMax
Protein common name
OmpA
Energy metabolism
Transport and binding proteins

Table 1 List of immunoreactive proteins of membrane proteins
Spot ID Gene symbol Protein common name NCBI GI identifier Cellular role
1 YaeT Hypothetical protein gi|30061734 Cell envelope
2 DnaK Chaperone Hsp70; autoregulated heat shock protein gi|30061584 Protein fate
3 ClpB Heat shock protein gi|30063993 Protein fate
4/14/16/17/20 OmpA Outer membrane protein 3a (II*; G; d) gi|30062494 Cell envelope
5 MopA GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein gi|30063518 Protein fate
6 Pgm Phosphoglucomutase gi|30062137 Energy metabolism
7 OppA Periplasmic oligopeptide binding protein gi|30062764 Protein fate
8/9 AtpA Membrane-bound ATP synthase, F1 sector, alpha-subunit gi|30094961 Energy metabolism
10 LpdA Lipoamide dehydrogenase (NADH) gi|30061682 Energy metabolism
11 Gnd Glucosamine-6-phosphate dehydrogenase gi|30063478 Energy metabolism
12/13/18 Tsf Protein chain elongation factor EF-Tu gi|30047373 Protein synthesis
15 Tsf Protein chain elongation factor EF-Ts gi|30061727 Protein synthesis
19 MglB Galactose-binding transport protein; receptor for galactose taxis gi|30063593 Transport and binding proteins

REFERENCES

1 Ahmed F, Ansaruzzaman M, Haque E, Rao MR, Clemens JD. Epidemiology of postshigellosis persistent diarrhea in young children. Pediatr Infect Dis J 2001; 20: 525-530
2 Liao X, Ying T, Wang H, Wang J, Shi Z, Feng E, Wei K, Wang Y, Zhang X, Huang L, Su G, Huang P. A two-dimensional proteome map of Shigella flexneri. Electrophoresis 2003; 24: 2864-2882
3 Humphrey-Smith I, Guyonnet F, Chastel C. Polypeptide cartography of Spiroplasma taiwanense. Electrophoresis 1994; 15: 1212-1217
4 Cordwell SJ. Acquisition and archiving of information for bacterial proteomics: from sample preparation to database. Methods Enzymol 2002; 358:207-227
5 Wu M, Stockley PG, Martin WJ. An improved western blotting technique effectively reduces background. Electrophoresis 2002; 23: 2373-2376
6 Liao X, Ying TY, Wang HL, Wang J, Wei KH, Huang LY, Huang PT. A modified method of in-gel digestion of Coomassie brilliant blue-stained 2-D gels. Shengwu Jishu Tongxun 2003; 14: 509-511
Sanchez-Campillo M, Bini L, Comanducci M, Raggiaschi R, Marzocchi B, Pallini V, Ratti G. Identification of immunoreactive proteins of Chlamydia trachomatis by Western blot analysis of a two-dimensional electrophoresis map with patient sera. *Electrophoresis* 1999; 20: 2269-2279

Bini L, Sanchez-Campillo M, Santucci A, Magi B, Marzocchi B, Comanducci M, Christiansen G, Birkeland S, Cevenini R, Vretou E, Ratti G, Pallini V. Mapping of Chlamydia trachomatis proteins by immobiline-polyacrylamide two-dimensional electrophoresis: spot identification by N-terminal sequencing and immunoblotting. *Electrophoresis* 1996; 17: 185-190

Pardo M, Ward M, Pitarch A, Sánchez M, Nombela C, Blackstock W, Gil C. Cross-species identification of novel Candida albicans immunogenic proteins by combination of two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Electrophoresis* 2000; 21: 2651-2659

McAtee CP, Lim MY, Fung K, Velligan M, Fry K, Chow T, Berg DE. Identification of potential diagnostic and vaccine candidates of Helicobacter pylori by two-dimensional gel electrophoresis, sequence analysis, and serum profiling. *Clin Diagn Lab Immunol* 1998; 5: 537-542

Haas G, Karaali G, Ebermayer K, Metzger WG, Lamer S, Zimny-Arndt U, Meyer TF, Aebischer T, Jungblut PR. Immunoproteomics of Helicobacter pylori infection and relation to gastric disease. *Proteomics* 2002; 2: 313-324

Nikaido H. Outer membrane. In: Neidhardt FC. *Escherichia coli and Salmonella: Cellular and molecular biology*. Washington DC: ASM Press, 1996: 29-47

Molloy MP, Herbert BR, Slade MB, Rabilloud T, Nouwens AS, Williams KL, Gooley AA. Proteomic analysis of the Escherichia coli outer membrane. *Eur J Biochem* 2000; 267: 2871-2881

Deb DK, Dahiya P, Srivastava KK, Srivastava R, Srivastava BS. Selective identification of new therapeutic targets of Mycobacterium tuberculosis by IVIAT approach. *Tuberculosis* 2002; 82: 175-182

Herbert M, Kraiss A, Hilpert AK, Schöhr S, Reidl J. Aerobic growth deficient Haemophilus influenzae mutants are non-virulent: implications on metabolism. *Int J Med Microbiol* 2003; 293:145-152

Ugalde JE, Comerchi DJ, Leguizamón MS, Ugalde RA. Evaluation of Brucella abortus phosphoglucomutase (pgm) mutant as a new live rough-phenotype vaccine. *Infect Immun* 2003; 71: 6264-6269

Sutcliffe IC, Russell RR. Lipoproteins of gram-positive bacteria. *J Biol Chem* 1997; 272: 15607-15612

Higgins CF, Hardie MM. Periplasmic protein associated with the oligopeptide permeases of Salmonella typhimurium and Escherichia coli. *J Bacteriol* 1983; 155: 1434-1438

Fenno JC, Tamura M, Hannam PM, Wong GW, Chan RA, McBride BC. Identification of a Treponema denticola OppA homologue that binds host proteins present in the subgingival environment. *Infect Immun* 2000; 68: 1884-1892

Richarme G, Caldas TD. Chaperone properties of the bacterial periplasmic substrate-binding proteins. *J Biol Chem* 1997; 272: 15607-15612

Robb CW, Orihuela CJ, Ekkelenskamp MB, Niesel DW. Identification and characterization of an in vivo regulated D15/Oma87 homologue in Shigella flexneri using differential display polymerase chain reaction. *Gene* 2001; 262: 169-177