

## Proteome Analysis of *Neisseria meningitidis* Serogroup C Strains Associated with Outbreaks in China<sup>1</sup>

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**Objective** During 2003-2005, an outbreak of meningitis due to *Neisseria meningitidis* serogroup C occurred in China. With the aim to find strain clues result in the final epidemics, the ancestral strain 053442, a clinical isolate, and a carrier strain 053426 with different gene type were analyzed. **Methods** Clinical strain 053442 and carrier strain 053426 were cultured on GC agar plates under the same condition. Two-dimensional electrophoresis was performed using the pH 3–10 nonlinear IPG strips of 24 cm length, and all the protein spots were identified by matrix-assisted laser desorption/ionization time of flight spectrometry. **Results** 502 and 380 protein spots were identified in 053426 and 053442 respectively, relating to 266 and 202 different genes covering a wide range of cellular functions. The express volume and number of proteins involved in energy metabolism, protein synthesis and amino acid biosynthesis in 053426 were higher than in 053442. Virulence factor Opa, Opc and a series of proteins involved in pilus assembly and retraction were identified in 053442, which appear to be of primary importance in colonization and invasion of human cells. Compared to 053442, virulence protein species were less in 053426, with lower express volumes too. No Opa and Opc were detected in 053426. **Conclusion** The different protein expression profiles of the clinical strain 053442 and carrier strain 053426 in the present study provide some clues of the different pathogenicity of the two strains, which may account for result in the final epidemics.

**Key words:** *Neisseria meningitidis*; Reference map; Two-dimensional gel electrophoresis; MALDI-TOF/TOF-MS

### INTRODUCTION

*Neisseria meningitidis* is the leading cause of bacterial meningitis and septicemia in children and young adults worldwide. According to the chemical composition of the capsular polysaccharides surrounding the bacterial cells, *N. meningitidis* strains are classified into 13 serogroups (A, B, C, D, E, H, I, K, L, W-135, X, Y, and Z), however, most cases of meningococcal disease are caused by only 5 serogroups (A, B, C, W-135, and Y)<sup>[1]</sup>.

In China, serogroup A *N. meningitidis* (menA) are responsible for more than 95% of cases, whereas serogroups B and C (menB and menC) cause only sporadic cases<sup>[2]</sup>. However, there was a sudden increase in the cases caused by menC strains during the meningitis outbreaks in 2003-2004 (five

outbreaks with 43 cases) and 2004-2005 (five outbreaks with 29 cases) in Anhui Province, China<sup>[3]</sup>. Many cases were found in other provinces in China at the same time. Multilocus sequence typing (MLST) results indicated that the unique sequence type 4821 (ST-4821) clone *N. meningitidis*, a new hyper-virulent lineage, was responsible for the menC meningitis outbreaks<sup>[3-4]</sup>. A nationwide survey showed that ST-4821 was the predominant genotype, accounting for 75% of the menC genotypes found in 12 provinces of China<sup>[5]</sup>.

The aim of this study was to find strain expression clues resulting in the hyper-virulent of ST-4821. The ancestral strain 053442 (ST-4821) resulted in the final epidemics in Anhui, and a carrier strain 053426 (ST-4837) isolated during this epidemic period were selected for future proteomic

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analysis in this study.

In 2000, genome sequencing of menA strain Z2 491 and menB strain MC58 were completed<sup>[6-7]</sup>. In 2004, differences in protein expression profiles were revealed by the proteome analysis of menA strain Z4 970 and menB strain MC58<sup>[8-9]</sup>. Genome sequencing of menC strain FAM18 and 053442 was recently completed<sup>[10]</sup>. The present study provided the first global proteome analysis and comparison of menC clinical and carrier strains isolated during the same epidemic. In addition, the results obtained in the present study were further compared with the previously published proteome data of the menA strain Z4 970 and menB strain MC58<sup>[8-9]</sup>.

## MATERIALS AND METHODS

### *Bacterial Cultures and Sample Preparation*

The menC clinical strain 053442 (ST-4821), isolated from the cerebrospinal fluid (CSF) of a patient in Anhui Province in 2004, and menC carrier strain 053426 (ST-4837), isolated from throat swab of an asymptomatic host in the same province in 2004<sup>[11]</sup>, were grown to a confluence on GC agar plates (Oxoid, Basingstoke, UK) plus supplements at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Bacteria were harvested from three plates, washed three times in phosphate-buffered saline (PBS), and the pellets were suspended in 20% trichloroacetic acid (TCA) in acetone containing 0.2% DTT and kept at -20 °C overnight. Following centrifugation (5 000 g, 10 min, 4 °C), the supernatant was discarded and the pellet resuspended in acetone containing 0.2% DTT. The sample was spun again, the supernatant discarded and the pellet was lyophilized under vacuum and then solubilized by sonication in lysis buffer (6 M urea, 2 M thiourea, 1% w/v DTT, 4% CHAPS, 2% IPG buffer pH 3–10 NL) and centrifuged at 18 000 g, 4 °C for 10 min. Protein concentration in the sample was determined by the Bradford method, with bovine serum albumin as a standard.

### *Two-dimensional Gel Electrophoresis*

The first dimension, isoelectric focusing (IEF), of two-dimensional gel electrophoresis (2-DE) was performed on a Pharmacia Biotech IPGphor Electrophoresis System. IPG DryStrips (nonlinear pH 3-10, 24 cm, GE Healthcare, Uppsala, Sweden) were rehydrated in the rehydrating buffer (6 M urea, 2 M thiourea, 1% DTT, 4% CHAPS, 0.5% IPGbuffer) with protein sample (1 mg and 200 µg, respectively, for preparative and analytical 2-DE). The IEF program was as follows: 30 V for 5 h, 60 V for 5 h,

100 V for 0.5 h, 300 V for 1 h, 600 V for 1 h, 1 000 V for 1 h, and 8 000 V for 10 h. After IEF, the strips were equilibrated in 0.375 M Tris buffer (pH 8.8) containing 6 M urea, 2% SDS, 20% glycerol, 2% dithiothreitol, and 0.01% bromophenol blue, followed by the adding of the same buffer supplemented with 2.5% iodoacetamide. The second dimension was performed on precasted 12.5% polyacrylamide gels at 25 °C in a Ettan™ DALTwelve System (GE Healthcare, Uppsala, Sweden). A protein molecular weight marker (Fermentas, SM0431) was applied on a corner of the gel to determine the relative molecular masses of proteins. The second dimension electrophoresis program was as follows: 2.5 W per gel for 0.5 h, 18 W per gel for 4.5 h (all were set for a constant watt).

### *Protein Visualization and Analysis*

The gel was loaded with 1 mg and 200 µg of protein sample followed by Coomassie Brilliant Blue staining and silver staining, respectively. Gels were digitized by scanning with Pharmacia Biotech ImageScanner, and analyzed by ImageMaste™ 5.0 2D platinum analysis software.

### *In-gel Digestion*

Spots from 2-DE were excised from the gel and placed in 96-well plate. Gel particles were washed with 25 mmol/L NH<sub>4</sub>HCO<sub>3</sub> for 20 min and destained with 30% acetonitrile in 100 mmol/L NH<sub>4</sub>HCO<sub>3</sub> three times (30 min each). Proteins were in-gel reduced with 10 mmol/L DTT in 100 mmol/L NH<sub>4</sub>HCO<sub>3</sub> for 60 min at 56 °C and S-alkylated with 55 mmol/L iodoacetamide in 100 mmol/L NH<sub>4</sub>HCO<sub>3</sub> for 30 min, in the dark. Gel particles were washed with 25 mmol/L NH<sub>4</sub>HCO<sub>3</sub>, dried under vacuum for 30 min, and then rehydrated with the digestion solution (40 µg/mL of trypsin in 25 mmol/L NH<sub>4</sub>HCO<sub>3</sub>). After incubation for 30 min at 4 °C, supernatants were replaced by 25 mmol/L NH<sub>4</sub>HCO<sub>3</sub>, and gel particles were incubated overnight at 37 °C.

### *MALDI-TOF/TOF-MS*

The samples were mixed (1:1) with a saturated matrix solution ( $\alpha$ -cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile and 0.1% formic acid). Mass spectra were obtained on a 4 700 Proteomics Analyzer with TOF/TOF optics (Applied Biosystems, Foster City, CA, USA) in the positive ion reflector mode with a mass accuracy of about 50 ppm. The MALDI tandem mass spectrometer equipped with a 200 Hz frequency-tripled Nd:YAG laser operating at a wavelength of 355 nm. MS spectra were obtained in the mass range between 800 and 4 000 Da with ca.

1 000 laser shots. MS/MS spectra were acquired with 2 000 laser shots using air as the collision gas. The singly charged peaks were analyzed using an interpretation method present on the instrument software, where the five most intense peaks were selected and MS/MS spectra were generated automatically, excluding those from the matrix due to trypsin autolysis peaks.

*Database Searches*

Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems, Foster City, CA, USA), with an internal Mascot V2.0 software (Matrix Science, UK) for searching the peptide mass fingerprints and MS/MS data. Searches were performed against the NCBI non-redundant protein database. Identifications with a GPS confidence interval of greater than 95% were accepted. The computational similarity search of each identified protein in the menA Z2491, menB MC58, menC FAM18, and menC 053442 genome was performed using the BLAST network service, whereas the theoretical molecular mass and pI values of the identified proteins were calculated from their predicted amino acid sequences in menC 053442 genome by using the ProtParam tool, both via the ExPASy website ([http:// au.expasy.org/cgi-bin/blast.pl](http://au.expasy.org/cgi-bin/blast.pl) and [http:// www.expasy.ch/tools/protparam.html](http://www.expasy.ch/tools/protparam.html), respectively). Experimental pI and molecular weight were automatically determined by bilinear interpolation between landmark features on each image.

**RESULTS**

*Proteome Data of MenC Isolates 053442 and 053426*

By using ImageMaste™ 5.0 2D platinum analysis software, 796 and 922 protein spots were detected from 2-DE maps of strains 053442 and 053426 respectively after Coomassie staining (Fig. 1 and Fig. 2). All the protein spots in the 2-DE maps were analyzed and 387 and 502 protein spots of strains 053442 and 053426 were identified respectively reflecting that 202 and 266 genes were expressed at the cellular abundance compatible with these staining procedures. When classified according to their cellular function, similar functional distribution obtained as shown in Fig. 3 confirms the same growth phase of the two strains. A total of 162 gene products were shared by the two strains that fall into 15 functional categories according to the four *N. meningitidis* genome annotations as shown in Table 1 and Fig. 4. The ones that were exclusively detected either in strain 053442 or in strain 053426

are shown in Table 2 and Table 3 respectively.

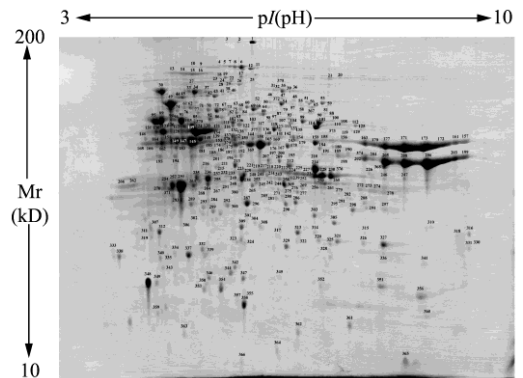


FIG. 1. Proteome map of clinical isolate 053442 of serogroup C *N. meningitidis*. Spot numbering refers to Table 1 and Table 2 which report protein identification by MS analysis.

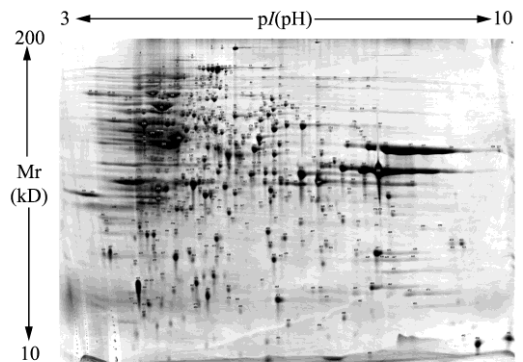


FIG. 2. Proteome map of carrier strain 053426 of serogroup C *N. meningitidis*. Spot numbering refers to Table 1 and Table 3 which report protein identification by MS analysis.

Tables 4 lists the 20 proteins with the highest spot volume in the 2-DE maps for strains 053442 and 053426 respectively accounting altogether for around 54.1% and 50.3% of the total spot volume.

*Comparison of The MenC Proteome Data with the Published MenA and MenB Proteome Data*

The first three proteins with the highest spot volume in the 053442 maps were outer membrane protein porA, porB, and rmpM, as well as in the 053426 map which differed from the proteome of the menA strain Z4970<sup>[8]</sup>. When classified according to their cellular function (Fig. 3), the most abundant proteins identified in the two menC strains were those involved in energy metabolism, amino acid biosynthesis, synthesis, and modification of macromolecules and outer membrane proteins, which was in good agreement with the reports of the menA and menB proteome data<sup>[8-9]</sup>.

For a more immediate comparison with data from the literature, all the counterpart gene codes in

menA, menB, and menC genome of each identified protein were exhibited in Tables 1-3 except for nine gene products, of which the counterpart genes were pseudogenes or absent in one or more of the four genomes. Most notable among them was spot 324 of strain 053426 which was identified as *N. gonorrhoeae* hypothetical protein NGO0702 that involved DNA methylation based on SwissProt annotation, but no homologous protein was found in the four genomes by BLAST.

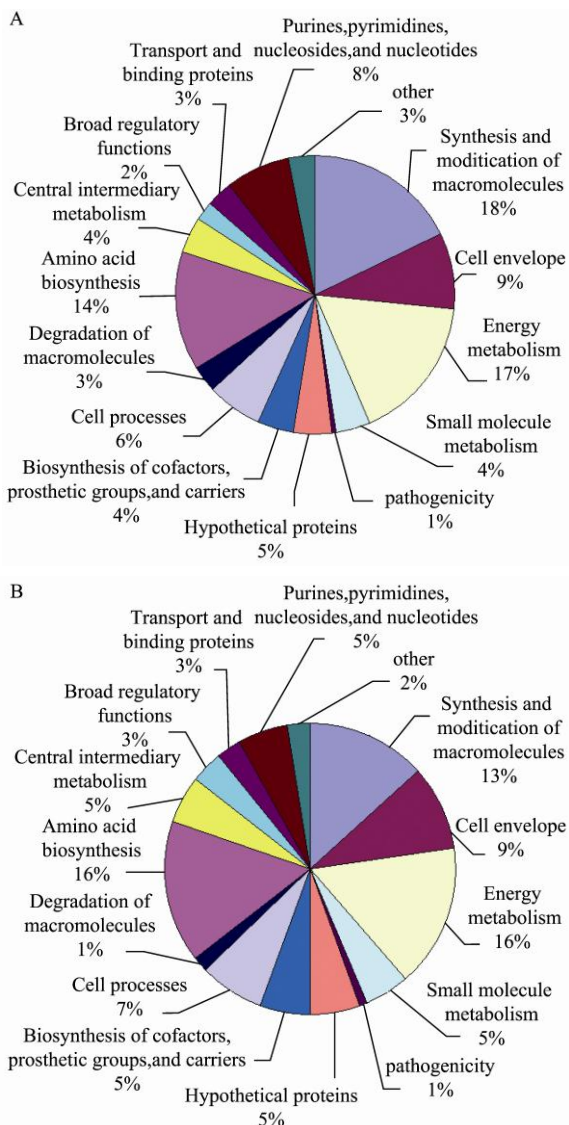


FIG. 3. Distribution of *N. meningitidis* proteins identified in menC carrier isolate 053426 (A) and clinical isolate 053442 (B) by 2-DE/MALDI-TOF, as per functional classification predicted on their genome sequence annotation.

For comparison of proteomic profiles of the menC strains 053442 and 053426 with the previously published proteomic data, each identified protein in

our study was marked in Tables 1-3 according to the detection achievement in the menA and menB proteomes<sup>[8-9]</sup>. 70 and 71 protein species, which represent 35% and 26.3% of all the species identified in 053442 and 053426 respectively, were overlapped with menA and menB proteomic data as shown in Fig. 5. A total of 409 gene products were identified in the four proteomic data, 65 of which were identified by all the 4 strains (shown in Table 1 by sigh + in both of the proteome A and B column). Almost all of these gene products had known functions that were necessary for survival, but with an exception for one protein (spot 354 for strain 053442 and spot 470 for strain 053426), which was identified as a hypothetical protein (NMCC\_1407).

All the tables and figures were available as supporting information on the web sites [http://www.hpbank.org/proteomebank/hy/Figure\\_1.pdf](http://www.hpbank.org/proteomebank/hy/Figure_1.pdf), [http://www.hpbank.org/proteomebank/hy/Figure\\_2.pdf](http://www.hpbank.org/proteomebank/hy/Figure_2.pdf), [http://www.hpbank.org/proteomebank/hy/Figure\\_3.pdf](http://www.hpbank.org/proteomebank/hy/Figure_3.pdf), [http://www.hpbank.org/proteomebank/hy/Figure\\_4.pdf](http://www.hpbank.org/proteomebank/hy/Figure_4.pdf), [http://www.hpbank.org/proteomebank/hy/Figure\\_5.pdf](http://www.hpbank.org/proteomebank/hy/Figure_5.pdf), [http://www.hpbank.org/proteomebank/hy/Table\\_1.pdf](http://www.hpbank.org/proteomebank/hy/Table_1.pdf), [http://www.hpbank.org/proteomebank/hy/Table\\_2.pdf](http://www.hpbank.org/proteomebank/hy/Table_2.pdf), [http://www.hpbank.org/proteomebank/hy/Table\\_3.pdf](http://www.hpbank.org/proteomebank/hy/Table_3.pdf), [http://www.hpbank.org/proteomebank/hy/Table\\_4.pdf](http://www.hpbank.org/proteomebank/hy/Table_4.pdf).

## DISCUSSION

Meningococcal disease occurs worldwide as endemic infections, and menA, menB, and menC *N. meningitidis* strains are responsible for the majority of the infections<sup>[11]</sup>. Our work is the first effort to compare expression profiles of menA, menB, and menC strains, which will be helpful for further meningococcal disease surveillance.

Both the numbers of protein spots and expressed genes detected in the carrier 053426 strain were more than those in clinical strain 053442 (Fig. 4), implying that the carrier strain needs to utilize more gene resources to support its survival. According to Tettelin *et al.*<sup>[6]</sup>, a crucial factor in the commensal and pathogenic behavior of *N. meningitidis* is its capacity to obtain and synthesize nutrients essential for survival. Under the same culture conditions, the carrier strain 053426 expresses more proteins, especially those involved in energy metabolism and protein synthesis, which implies that the strain cause disease maybe has the ability to obtain nutrients more efficiently.

The most attractive difference between 053426 and 053442 expression profiles is the cell envelope proteins, especially the outer membrane proteins involved in pathogenesis. Of the virulence factors that have been identified in pathogenic *Neisseria*, pili,

outer membrane adhesins/invasins Opa and Opc appear to be of primary importance in colonization and invasion of human cells<sup>[12-13]</sup>. In the present study, proteins involved in pilus assembly (PilO and PilN), pilus secretin (PilQ), pilus retraction (PilU, PilT, and PilT2) and a pilE regulator (RegF) were detected, demonstrating that after the establishment of initial pilus-mediated adhesion, both the 053442 and 053426 strains have the ability to retract pilin to trigger a more intimate adhesion. Among them, PilO and PilT were not detected in the proteome of 053442 and 053426 respectively.

During the infection process, integral outer membrane adhesins/invasins Opa and Opc proteins, are important to establish an intimate association with the human cells. Both Opa and Opc were identified in the clinical strain 053442, but not in the carrier strain 053426. Opa proteins are highly diverse, and undergo high-frequency phase and antigenic variation<sup>[14-15]</sup>. The genomes of menA Z2491 and menB MC58 contain 3 and 4 variant *opa* genes, respectively, but all are inactivated by a frameshift caused by the pentameric repeats (CTCTT) region within the signal sequence-encoding segments<sup>[16]</sup>. The menA and menB proteomes failed to detect the Opa protein too<sup>[8-9]</sup>. In the present study, Opa protein was present

as multiple spots in the 2-DE map of the strain 053442, with variability in both pI values and apparent Mr (spot 267, 268, 336-339 in Fig. 1), indicating that more than 2 Opa variations were expressed. According to Virji *et al.*, different Opa variants exhibit different interactions with human cells<sup>[17]</sup>. Particular combinations of Opa proteins confer fitness advantages for transmission from host to host<sup>[18]</sup>. Therefore, the Opa repertoires of strain 053442 provide us a clue to investigate the pathogenicity and transmission ability of strain further.

Opc is another outer membrane protein that is important for the pathogenesis of meningococcal disease. The *opc* gene is contained by many, but not all *N. meningitidis* strains<sup>[19]</sup>. Opc also undergoes phase variation due to the transcriptional regulation. The transcription of *opc* may vary from zero to the intermediate or high level by size variation of a promoter containing poly-cytidine<sup>[20]</sup>. Opc significantly increases adhesion and invasion of bacteria into both endothelial and epithelial cells when present at high levels<sup>[17, 21]</sup>. In the present study, Opc was expressed with the eighth highest spot volume in the 2-DE map of the strain 053442 grown on agar plates without specific environmental stimuli (Table 4), implying that the increased expression of Opc may be one of

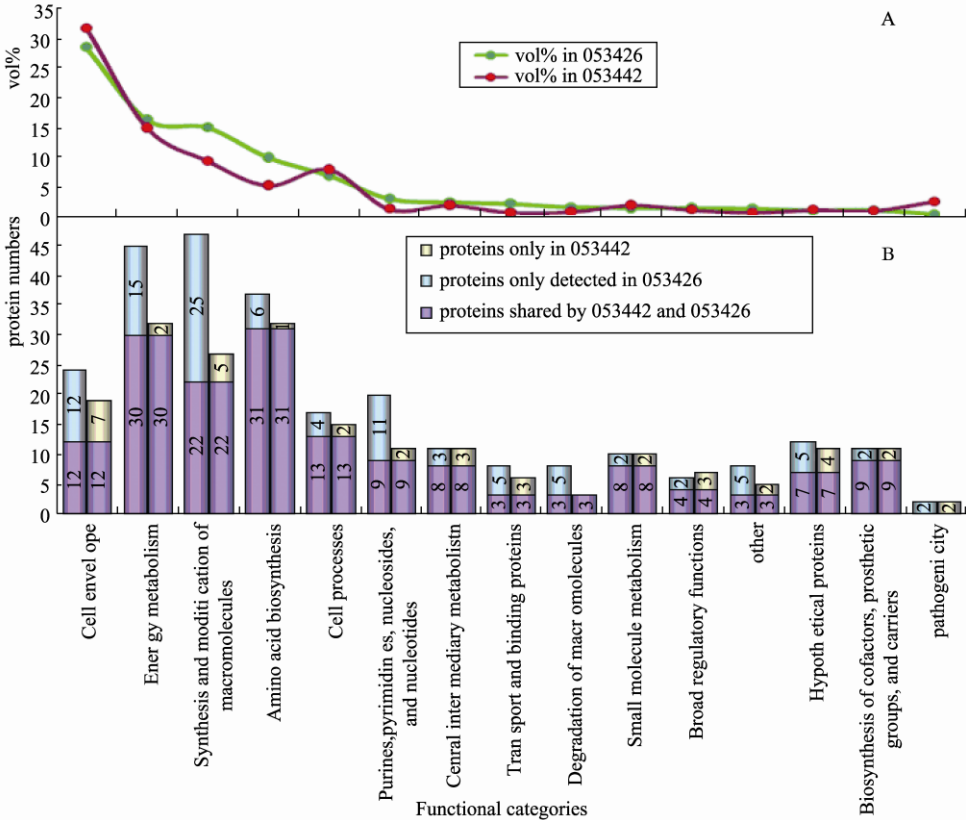


FIG. 4. Comparison of total spot volume in 2-DE map of proteins identified in menC carrier isolate 053426 and clinical isolate 053442, classified by the protein function (A). The numbers in the bar graph are the sum of identified proteins belonging to each functional classification (B).

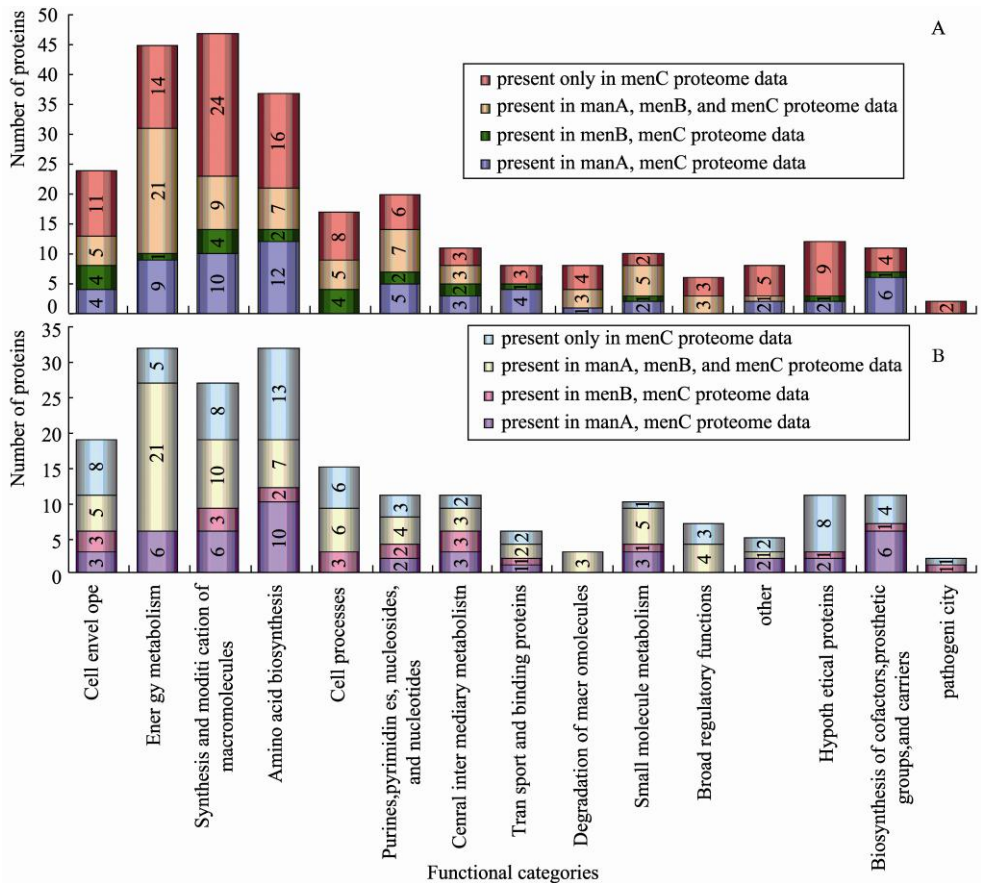


FIG. 5. Comparison of proteins identified in menC carrier isolate 053426 (A) and clinical isolate 053442 (B) proteome data with the published menA and menB proteome researches 7, 8. The numbers in the bar graph are the sum of identified proteins belonging to each functional classification.

the reasons for the hyper-invasiveness of the strain 053442. It has been reported that Opc is more effective in mediating invasion of endothelial cells than in epithelial cells<sup>[22]</sup>, and is needed for the interaction with endothelial cells of the blood-brain barrier<sup>[23]</sup>. The clinical strain 053442 was isolated from a patient's CSF. The ability of 053442 to cross the blood-brain barrier may be induced by the high Opc expression level too. These findings suggest that not only *opc* gene expression but also the expression level should be detected in the future molecular epidemiological studies of *N. meningitidis* strains and the associated disease surveillance.

Although Opa and Opc were not detected, the carrier strain 053426 expressed an adhesin complex protein (gene locus tag was NMCC\_2060 in 053442 genome) that was not identified in the clinical strain 053442 (Table 3). No contribution of this adhesin protein to the adhesion or colonization has been reported yet. In addition, a PmbA protein, which has been reported to be required for the production of the

antibiotic peptides in *Escherichia coli*<sup>[24]</sup>, was identified in the strain 053426, but not in the strain 053442. If *N. meningitidis* PmbA has the same function, expression of PmbA protein must be helpful for the carrier strain 053426 to colonize the nasopharyngeal mucosa by killing or suppressing other commensals.

Overall, the *N. meningitidis* carrier and disease development is very complicated depending on the balance amongst the strain, the host, and the microenvironment of the nasopharynx. The strain factors that trigger infection disease is poorly understood now. The protein expression profiles of the clinical strain 053442 and carrier strain 053426 in the present study provide some clues to the final epidemics. The different gene repertoires and expression volume of the virulence factors revealed by the proteome analysis will be helpful for epidemiological investigation and surveillance of meningococcal disease and will improve our understanding of the pathogenesis of *N. meningitidis*.

TABLE 4

The Twenty *N. meningitidis* Proteins with the Highest Volume in Serogroup C Clinical Isolate 053442 and Carrier Strain 053426 2-DE Maps

| No. | 053442                                      |       |           |   |       | 053426  |       |           |   |       |
|-----|---|-------|-----------|---|-------|---|-------|-----------|---|-------|
|     | Protein Name                                | Gene  | Proteome* |   | vol%  | Protein Name  | Gene  | Proteome* |   | vol%  |
|     |   |       | A         | B |       |   |       | A         | B |       |
| 1   | porin, class I outer membrane protein       | porA  | +         | + | 13.3  | porin, major outer membrane protein P.I                           | porB  | +         | + | 10.8  |
| 2   | porin, major outer membrane protein P.I     | porB  | +         | + | 11.6  | porin, class I outer membrane protein                             | porA  | +         | + | 9.7   |
| 3   | outer membrane protein class 4              | rmpM  | +         | + | 5.2   | outer membrane protein class 4                                    | rmpM  | +         | + | 4.6   |
| 4   | elongation factor Tu                        | tufA1 | +         | + | 3.1   | cysteine synthase   | cysK  | +         | + | 4.0   |
| 5   | peroxiredoxin 2 family protein/glutaredoxin |       | +         | + | 2.7   | succinyl-CoA synthetase subunit alpha                             | sucD  | +         | + | 2.2   |
| 6   | alcohol dehydrogenase                       | adhP  | +         | + | 2.6   | chaperonin GroEL  | groEL | +         | + | 2.1   |
| 7   | succinyl-CoA synthetase subunit alpha       | sucD  | +         | + | 1.7   | elongation factor Tu  | tufA1 | -         | + | 1.8   |
| 8   | outer membrane protein precursor            | Opc   | -         | + | 1.6   | bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase | acnB  | +         | + | 1.7   |
| 9   | succinyl-CoA synthetase subunit beta        | sucC  | +         | + | 1.6   | alcohol dehydrogenase   | adhP  | +         | + | 1.3   |
| 10  | cysteine synthase                           | cysK  | +         | + | 1.6   | dihydrolipoamide dehydrogenase E3 component                       | lpdA3 | +         | + | 1.2   |
| 11  | chaperonin GroEL                            | groEL | +         | + | 1.3   | 50S ribosomal protein L25   | rplY  | +         | + | 1.2   |
| 12  | 50S ribosomal protein L9                    | rpII  | -         | + | 1.1   | peroxiredoxin 2 family protein/glutaredoxin                       |       | +         | + | 1.2   |
| 13  | phosphate acetyltransferase                 |       | +         | + | 1.1   | F0F1 ATP synthase subunit alpha                                   | atpA  | +         | + | 1.2   |
| 14  | dihydrolipoamide dehydrogenase E3 component | lpdA3 | +         | + | 0.9   | trigger factor  | tig   | +         | + | 1.1   |
| 15  | elongation factor G                         | fusA  | +         | + | 0.9   | 50S ribosomal protein L7/L12                                      | rplL  | -         | + | 1.1   |
| 16  | molecular chaperone DnaK                    | dnaK  | +         | + | 0.9   | elongation factor TS  | tsf   | +         | + | 1.1   |
| 17  | F0F1 ATP synthase subunit beta              | atpD  | +         | + | 0.8   | amino acid permease substrate-binding protein                     |       | +         | - | 1.0   |
| 18  | cell division protein                       | ftsA  | +         | + | 0.7   | succinyl-CoA synthetase subunit beta                              | sucC  | +         | + | 1.0   |
| 19  | glutamate dehydrogenase                     | gdhA  | +         | + | 0.7   | acetate kinase  | ackA1 | +         | + | 1.0   |
| 20  | F0F1 ATP synthase subunit alpha             | atpA  | +         | + | 0.7   | 30S ribosomal protein S2  | rpsB  | +         | - | 1.0   |
|     | SUM   |       |           |   | 54.1% | SUM   |       |           |   | 50.3% |

Note. \*Positive sign (+) marks a protein detection achieved also in the menA (A) or menB (B) proteome; negative sign (-) marks a protein identification did not achieved in the menA or menB proteome.

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