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Proteomic study of non-typable *Haemophilus influenzae*

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Abstract

Non-typable *Haemophilus influenzae* (NTHi) are small, gram-negative bacteria and are strictly human pathogens, causing acute otitis media, sinusitis and community-acquired pneumonia. There is no vaccine available for NTHi, as there is for *H. influenzae* type b. Recent advances in proteomic techniques are finding novel applications in the field of vaccinology. There are several protein separation techniques available today, each with inherent advantages and disadvantages. We employed a combined proteomics approach, including sequential extraction and analytical two-dimensional polyacrylamide electrophoresis (2D PAGE), and two-dimensional semi-preparative electrophoresis (2D PE), in order to study protein expression in the A4 NTHi strain. Although putative vaccine candidates were identified with both techniques, 11 of 15 proteins identified using the 2D PE approach were not identified by 2D PAGE, demonstrating the complementarily of the two methods.

Keywords: Proteomics; Haemophilus influenzae

1. Introduction

Haemophilus influenzae is a, small, non-motile, pleomorphic, gram-negative organism. The bacterium is a strict human pathogen, colonizing the upper respiratory tract. It can become invasive by penetrating the nasopharyngeal epithelial barrier and entering the blood stream. Encapsulated *H. influenzae* type b mainly cause meningitis in children, but fortunately, an effective vaccine is available.

Non-typable *H. influenzae* (NTHi), which are non-encapsulated, cause acute otitis media, sinusitis and community-acquired pneumonia [1]. The development of an effective vaccine for the prevention of NTHi infection has proved difficult. The reasons for this include: (1) genetic variability of NTHi strains as compared to the published genome of *H. influenzae* Rd, a type b strain [2]; (2) antigenic variability in major outer membrane proteins [3]; as well as (3) the technical difficulties of identifying and characterizing adhesins and outer membrane proteins, which are important targets for vaccine development [4]. NTHi strains are no longer viewed as non-encapsulated versions of *H. influenzae* type b. Indeed, a study of the genetic relationships of 177 type b and 65 NTHi clinical isolates indicated that NTHi are both genetically distinct from type b and genetically more diverse [5]. The major outer membrane proteins of NTHi are immunogenic, but due to interstrain variability, these have not been found to be effective vaccine components [3].

Until recent years, the development of vaccines

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relied on the elicitation of an immune response to attenuated or whole-cell preparations, or empirically chosen antigens. The post-genomic era of bacteriology holds the possibility of rational vaccine design. The discovery and development of new vaccines is likely to be achieved through integrated genomic and proteomic strategies [6]. Analytical two-dimensional polyacrylamide gel electrophoresis (2D PAGE) [7] is by far the most widely employed separation technique for proteomic studies. The separating ability of 2D PAGE is high and reproducible. Proteins can be quantitated and certain post-translational modifications readily identified. The genome of *H. influenzae* has been well characterized through 2D PAGE, where a large number of predicted proteins have been identified [8,9]. Indeed, the largest number of proteins identified by 2D PAGE to date occured in a study of a strain of *H. influenzae*, and covered about one-third of the predicted proteome [10].

The 2D PAGE technique is, however, difficult to use for studies of membrane and low copy number proteins [11]; these are frequently the targets of researchers in the field of vaccinology. Considerable efforts have been devoted to improving 2D gel protocols for studies of membrane proteins [12–15] through the use of sample fractionation and differential solubilization [16], with some success. However, the limitations of 2D PAGE have lead investigators to develop techniques which provide complementary information [11]. One of these techniques, two-dimensional semi-preparative electrophoresis (2D PE), has been applied in the study of solubilized proteins from *Helicobacter pylori* [17] and *Escherichia coli* [18], resulting in the identification of a high number of membrane-, membrane-associated and low abundance proteins. The technique is based on the same isoelectric focusing and gel electrophoretic principles as analytical 2D PAGE [19]. In the first dimensional separation, proteins are enriched up to 500 times in liquid fractions at their respective isoelectric points. In the second dimension, IEF-enriched proteins are separated by SDS–PAGE, then eluted from the gel into liquid fractions. Because high amounts of proteins (up to 1 g) can be separated, low abundance proteins are not discriminated against in 2D PE. However, 2D PE does not provide visualization or quantitation of proteomes, or the high resolution of separation of 2D PAGE. The 2D PE technique has been suggested to be useful as a complementary tool to 2D PAGE in proteomic investigations [11], but the two techniques have not previously been employed in parallel in the same study. Therefore, in our investigation of the proteome of an NTHi strain, we employed both sequential extraction of proteins followed by 2D PAGE and 2D PE of a solubilized NTHi bacterial pellet, followed by enzymatic digestion, mass spectrometry and database searches.

2. Experimental

2.1. Bacterial cultivation and protein preparation

NTHi strain A4 was cultivated on chocolate agar plates over night at 37 °C in a low partial pressure of oxygen. The bacteria from two plates (approximately 3 g wet weight each) were harvested and the pellets were washed in PBS.

2.2. Analytical 2D PAGE

2.2.1. Sequential extraction and whole cell extraction of NTHi

Proteins from one culture dish were extracted from NTHi according to the instructions of the ReadyPrep™ Sequential Extraction Kit from BioRad, which is based on a method described by Molloy et al. [16]. In the first step (extraction 1), the bacterial pellet was dissolved in Reagent 1 (40 m*M* Tris base) followed by tip sonication on ice at an amplitude of 4 μm 6×10 s with 10 s pauses. Endonuclease (150 u/ml) was added and the mixture was incubated for 15 min at 37 °C. The bacterial lysate was centrifuged for 10 min at maximum speed in a bench-top microcentrifuge in order to pellet insoluble materials. The supernatant was recovered (supernatant 1) and the remaining pellet was washed three times in Reagent 1. The pellet was dissolved in Extraction Solution 2 (2 m*M* tributylphosphine in Reagent 2: 8 *M* Urea, 4% (w/v) CHAPS, 40 m*M* Tris, 0.2% (w/v) Bio-Lyte 3/10) and vortexed for 5 min. After 10 min of centrifugation the supernatant (supernatant 2) was recovered. The remaining insoluble pellet was washed two times in Extraction Solution 2. To this pellet, Extraction Solution 3 (2 m*M* TBP in Reagent 3: 5 *M* Urea, 2 *M* Thiourea, 2% (w/v) CHAPS, 2%

SB 3-10, 40 m*M* Tris, 0.2% (w/v) Bio-Lyte 3/10) was added. The mixture was vortexed and centrifuged as before and the supernatant was recovered (supernatant 3). Protein concentrations were estimated using the BCA Protein Assay Reagent (Pierce) for supernatant 1 and the Coomassie Plus Protein Assay Reagent (Pierce, USA) for supernatant 2, and 3.

2.2.2. In-gel isoelectric focusing

IPG strips, 11 cm, with linear pH 3–10 gradient (Immobiline™ Dry Strip gels, Amersham Pharmacia Biotech), were rehydrated at room temperature in a total sample volume of 200 μl including 200 μg sample and rehydration buffer (Extraction Solution 2 (supernatant 1), Extraction Solution 3 (supernatant 2, 3 and supernatant from whole cell extraction), 0.5% IPG Buffer pH 3–10, bromophenol blue. Rehydration was performed at 30 V for 15 h followed by IEF with the following steps: (1) 200 V for 1 h; (2) 300 V for 1 h; (3) 500–8000 V for 1 h; and (4) 8000 V for 4 h (IPGphor™ Isoelectric Focusing System, Amersham Pharmacia Biotech). The strips were equilibrated for 15 min in 2.5 ml SDS equilibration buffer (50 m*M* Tris–HCl pH 8.8, 6 *M* Urea, 30% glycerol, 2% SDS, bromphenol blue, 2 m*M* tributylphosphine (TBP). The equilibration procedure was repeated once more in SDS buffer without the addition of TBP.

2.2.3. SDS–PAGE

The IPG strips were placed on the top surface of 10% Tris–HCl gels (Criterion, BioRad) and sealed with 0.5% agarose in gel buffer. Running buffer (192 m*M* glycine, 25 m*M* Tris, 0.1% SDS) was added and the separation proceeded for 55 min. The gels were washed in ultrapure water 3×10 min and stained with Colloidal Coomassie Blue (Gel Code Blue™, Pierce).

2.2.4. In-gel digestion of proteins

Protein spots were excised and placed in siliconized tubes. One hundred microlitres of a destaining solution containing 50% acetonitrile and 25 m*M* ammonium bicarbonate was added, and vortexed 20 min. After centrifugation, the supernatant was removed. The procedure was repeated twice. The samples were dried in a vacuum centrifuge for 30 min and 12 μl of trypsin (10 ng/μl) solution was added followed by incubation in 37 °C for 16 h. The mixture was centrifuged quickly and 12 μl 5% TFA, 75% acetonitrile, was added. The samples were vortexed for 30 min after which the tubes were stored at −20 °C.

2.3. Two-dimensional preparative electrophoresis

2.3.1. Liquid-phase isoelectric focusing

A second bacterial pellet was dissolved in 1 ml digitonin (0.1%) and ultrasonicated for 10 min (Soniprep 150, MSE). Another 14 ml of digitonin (0.1%) and Servalyte (40%, pH range 3–10 isodalt, Serva Electrophoresis, GmbH, Germany) was added to the sample at a final concentration of 0.8%. Liquid-phase IEF was then performed according to experimental procedures described in Davidsson et al. [19]. The sample was loaded in a pre-cooled Rotofor apparatus (Bio-Rad Laboratories, Hercules, CA) for fractionation over a wide range pH gradient (pH 3–10). Constant power (10 W) was applied to the system. The initial voltage applied was 880 V and protein separation was allowed to continue until a voltage plateau was reached, after about 2 h. Twenty fractions were harvested and pH was measured. Seventy-five microlitres of each fraction was analyzed with SDS–PAGE using the NuPAGE system, which includes 10% Bis–Tris gels, 3-(*N*-morpholino)-propane sulfonic acid (MOPS) sodium dodecyl sulphate (SDS) running buffer system. Proteins were detected using colloidal Coomassie blue (Gel Code Blue®, Pierce).

2.3.2. SDS–PAGE and electroelution

Liquid-phase IEF fractions containing the highest protein amounts were selected for further analysis. Fractions were concentrated by vacuum centrifugation and dissolved in 200 μl NuPAGE SDS sample buffer (Novex) and heated at 100 °C for 3 min. The sample was separated using the NuPAGE system run at 200 V for 55 min. Proteins were eluted from the gel using the mini whole gel eluter [19]. The system was run at 50 V.

Eluted fractions were dried to 200 μl. Six-hundred microlitres of ice cold acetone was added and the

samples were incubated at −20 °C for 2 h. The samples were centrifuged and dried after the supernatant was removed. The proteins were dissolved in 25 μl digestion buffer (0.1 m*M* $CaCl_2$, 0.1 *M* NH_4HCO_3) and 10 μl (10 ng/μl) of porcine trypsin (Promega Inc. Madison, WI, USA) dissolved in 25 m*M* NH_4HCO_3, was added followed by incubation at 37 °C for 4 h. The samples were dried and dissolved in 25 μl TFA (0.1%).

2.4. Mass spectrometry

2.4.1. MALDI-TOF-MS

Dried tryptic digests were reconstituted in 25 μl 0.1% TFA and treated with C_{18} Zip Tips™ (Millipore) according to the manufacturer's instruction. A sample (0.5 μl) was mixed with 0.5 μl matrix solution directly on the MALDI probe and allowed to dry at the ambient conditions. The matrix was α-cyano-4-hydroxy cinnamic acid, dissolved 10 mg/ml in acetonitrile–H_2O, 1:1. Peptide spectra were acquired in reflectron mode at an accelerating voltage of 20 kV. External calibration using angiotensin II and ACTH was used. MALDI-spectra were analyzed using the MassLynx™ software. Resulting values for monoisotopic peaks were used for searches (MS-Fit, http://prospector.ucsf.edu) against the NCBInr database. Species, p*I*, and molecular mass were unrestricted. A mass tolerance of +200 ppm was allowed.

2.4.2. ESI-qTOF-MS–MS

Samples that could not be identified with certainty by peptide mapping were analyzed by nanoflow electrospray tandem mass spectrometry in a Q-Tof (Micromass, UK). Samples were enriched using Zip Tips, eluted with acetonitrile–H_2O containing 0.1% formic acid, and sprayed from gold-coated glass capillaries (Micromass). The collision gas was argon. Instrument calibration was performed using fragment ions from Glu-fibrinopeptide B and a fourth-order polynormal fit. Fragment ion spectra were post-processed and saved in Sequest-compatible format and used to search the entire NCBI nr database with the MASCOT protein bioinformatics tool (www.matrixscience.com). A mass tolerance of ±200 ppm was allowed for the peptide precursor mass.

3. Results

3.1. Sequential protein extraction and 2D page

Proteins extracted sequentially were separated in three separate 2D gels and stained with Coomassie blue. The sequential extraction procedure is expected to yield the most hydrophilic proteins in supernatant 1 (Fig. 1A), hydrophobic proteins in supernantant 2 (Fig. 1B), and the most refractory hydrophobic proteins in supernatant 3 (Fig. 1C). Twelve spots were cut out from each of the gels for enzymatic digestion, mass spectrometry and database searches. Twenty-one protein identities were obtained (Table 1), most through peptide mapping. Some of the identified proteins, outer membrane protein 26, elongation factor Tu (tufB) and outer membrane protein P2 appeared in two or more spots in different gels. Four of the identified proteins in Table 1 are membrane-associated: fimA and outer membrane proteins P2, P4 and 26. Four other proteins, outer membrane protein 26, dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex E2, lysine-tRNA ligase, and hypothetical protein HI1004, have to the best of our knowledge, not been previously identified in proteomic studies of *H. influenzae*.

3.2. 2D PE

2D PE was performed on a digitonin-solubilized bacterial pellet from non-typable *H. influenzae*. Rotofor fractions 3, 10 and 12 (pH 2.5, 5.5 and 6 respectively) were chosen for further analysis. Proteins in these fractions were separated by SDS–PAGE, eluted into liquid fractions, and digested with trypsin prior to mass spectrometry. Through this strategy, fifteen protein identities were obtained (Table 2). The assignment of twelve of these identities required the acquisition of MS–MS data from the protein digests. Two of the identified proteins in Table 2 are hydrophobic, outer membrane protein P2 and 26. Three of the identified proteins, arginine-binding periplasmic protein precursor, fimbrial protein, and adenylate kinase, have to the best of our knowledge, not been identified previously in proteomic studies of *H. influenzae*. Remarkably, only four of the proteins identified in the 2D PE procedure

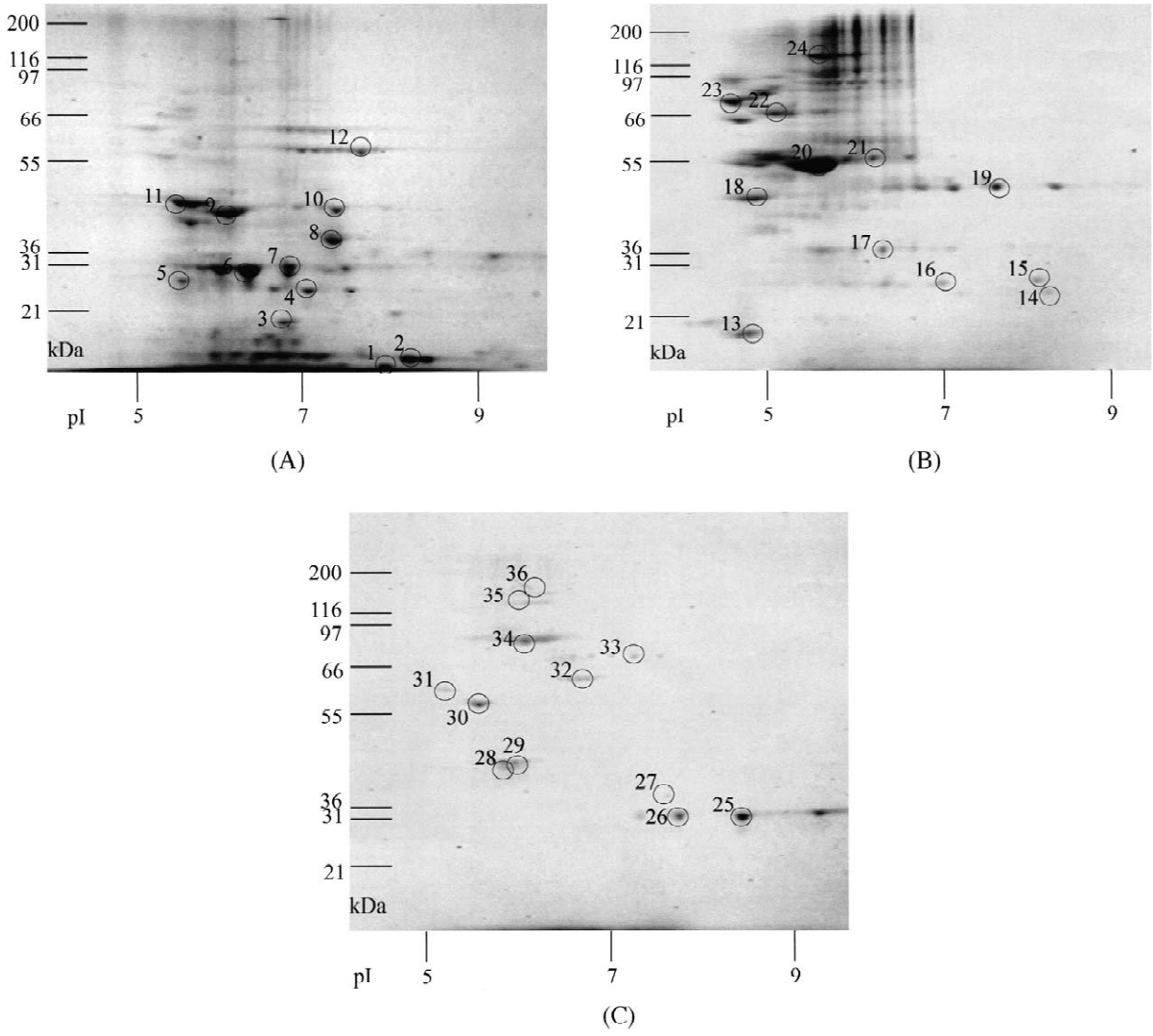

Fig. 1. Proteins from first, second and third extraction were separated on 2D PAGE using 11 cm IPG strips with linear pH of 3–10, followed by electrophoresis on 10% Tris–HCl 1.0 mm gels. Staining was performed with Coomassie blue. Spots 1–12 were excised from gel A (first extraction), spots 13–24 (second extraction) were excised from gel B and spots 25–36 (third extraction) were excised from gel C. The spots were digested with trypsin and identified with MALDI TOF MS and ESI-QTOF MS–MS. The names of the identified proteins are given in Table 1.

were also identified when 2D PAGE was employed as the separation technique.

4. Discussion

We chose to study the proteome of NTHi strain A4 with a combination of protein separation techniques, sequential extraction /2D PAGE and 2D PE. Our results demonstrate the complementary nature of the results obtained in each part. The procedure employing sequential extraction of proteins prior to separation by 2D PAGE was specifically developed to increase the number of hydrophobic proteins identified in 2D gels [16], and yields three different images of the part of the proteome that is soluble by

Table 1
Proteins identified in NTHi strain A4, separated by differential protein extraction/2D PAGE

Gel	Spot	Assignment	Accession no.	Molecular Mass (calc)	MOWSE score	Mascot score
1	2	Outer membrane protein 26[a]	4574254	22 089	3.47e+004	
	4	Conserved hypothetical protein	1175165	36 513	3.13e+003	
	5	Elongation factor Ts (tsf)	1169482	30 188	2.6e+004	
	6,7	D-galactose-binding protein	1073976	37 580		42
	8	Putative adhesin B precursor FimA	3003012	34 958	3.58e+003	
	9	Elongation factor Tu (tufB)	1169492	43 355	2.67e+006	
	10	HtrA	2935166	46 377	1.11e+004	
	11	Trigger factor (tig)	1174696	48 332	6.88e+004	
	12	5′-nucleotidase NucA precursor	4929317	66 242	3.01e+007	
2	14	Hypothetical protein HI1681 precursor	1176070	23 381		94
	15	Outer membrane protein 26[a]	4574254	20 208	1.36e+003	
	17	Outer membrane protein P4 precursor	97171	30 484		73
	18	Spermidine/putrescine-binding periplasmic protein 1 precursor (SPBP)	1172560	39 989	8.68e+004	
	19	Outer membrane protein P2	148963	42 915		55
	20,21,24	Elongation factor Tu (tufB)	1169492	43 355	8.11e+004	
	22	Ribosomal protein S1 (rpS1)	2500383	59 560	2.6e+004	
	23	Heat shock protein 70 (dnaK)	1169375	68 282	4.63e+008	
3	25	Outer membrane protein P2 precursor (OMP P2)	3914222	39 849		
	29	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (E2)[a]	1171887	45 135		58
	30	Lysyl-tRNA synthetase (Lysine-tRNA ligase)[a]	1174526	56 900		88
	31	Hypothetical protein HI1004[a]	1074600	66 545		85
	32	Transketolase 1 (tktA)	1174714	72 719	4.7e+003	
	34	Pyruvate dehydrogenase, E1 component (aceE)	1171888	99 132	2.41e+004	

[a] Protein not been previously identified in NTHi.

Table 2
Proteins identified in NTHi strain A4, separated by 2D PE

Assignment	Accession no.	Molecular Mass (calc.)	MOWSE score	Mascot score
50S Ribosomal protein L1	417663	24 092		206
D-galactose binding periplasmic protein precursor	1169276	35 496		109
Arginine-binding periplasmic protein precursor[a]	1168523	26 148		121
Phosphoenolpyruvate carboxykinase	1172573	59 651		448
Glyceraldehyde3-phosphate dehyrogenase	146093	33 175		60
Pyruvate dehydrogenase, E1 component,aceE	1171888	99 131	2.89E+007	
Elongation factor G	1169479	77 499		225
Elongation factor Tu	1169492	43 469	1.53+007	457
Major outer membrane protein P2-type 8	1881750	35 469		109
Outer membrane protein 26[a]	4574284	20 806		50
Fimbrial protein[a]	1171900	38 340	6.2E+005	
Hypothetical protein HI1647	1176057	31 732		48
Hypothetical protein HI1624 precursor	1176043	25 548		91
60 KD chaperonin[a]	1168916	57 683		78
Adenylate kinase[a]	1170608	23 564		67

[a] Protein not been previously identified in NTHi.

each extraction method. Using sequential extraction prior to analytical 2D PAGE, we were able to identify four membrane proteins out of a total of 20 identified proteins. The 2D PE procedure, in which whole bacteria are solubilized prior to separation, yielded only two membrane protein identities, and these proteins were also detected by 2D PAGE. The reason for this may be that other membrane proteins were separated into IEF fractions that were not analyzed further. Eleven out of 15 proteins identified when 2D PE was used as a separation technique were not identified by 2D PAGE, bringing the total number of identified proteins to thirty three.

Three of the detected proteins are putative vaccine candidates, OMP 26, OMP P4, and HtrA. Immunization with OMP 26 in an experimental animal model was found to be protective and induce an IgA antibody response [20]. The function of OMP P4 has been identified as a novel phosphomonoesterase [21]. Two previously described OMPs, P5 and P6, were not detected in this study. OMP P5 displays inter-strain variabilty in three hypervariable regions and is therefore not an ideal vaccine candidate [3]. OMP P6 was suggested to be a candidate vaccine protein through a bioinformatics approach and identified in a 2D gel [22].

It is possible that OMP P5 and P6 were expressed by NTHi strain A4, but not detected when either separation strategy was employed. However, proteins may be expressed in one bacterial isolate but be lacking in another. An illustration of this is the case of tryptophanase, which is expressed by a strain of *H. influenzae*, but is not found in the sequenced (Rd) strain [23]. Also, NTHi are known to undergo phase variation of pili and lipooligosaccharide composition; it is therefore possible that OMP P5 and P6 expression was lacking at the time of our study. This same line of reasoning may apply to the proteins identified in our study, that have not previously been described in proteomic investigations of *H. influenzae*.

During the past few years, more than forty complete prokaryotic genome sequences have been published. In vaccine discovery, it is important to selectively identify virulence proteins which may induce a protective immunological response in the host. Advances in techniques to separate and identify expressed proteins against genome sequences have been made, and these are finding use in the field of vaccinology. But because no single method can truly be claimed to provide global information about protein expression, the future success of projects aimed at rational vaccine design is likely to depend on combined approaches.

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References

[1] V.K. Rao, G.P. Krasan, D.R. Hendrixson, S. Dawid, J.W. St Geme 3rd, FEMS Microbiol. Rev. 23 (1999) 99.
[2] R.D. Fleischmann, M.D. Adams, O. White, R.A. Clayton, E.F. Kirkness, A.R. Kerlavage, C.J. Bult, J.F. Tomb, B.A. Dougherty, J.M. Merrick, K. McKenny, G. Sutton, W. Fitzhugh, C.A. Fields, J.D. Gocayne, R. Shirley, L.-I. Liu, A. Glodek, J.M. Kelley, J.F. Weidman, C.A. Phillips, T. Spriggs, E. Hedblom, M.D. Cotton, T.R. Utterback, M.C. Hanna, D.T. Nguyen, D.M. Saudek, R.C. Brandon, L.D. Fine, J.L. Fritchman, J.L. Fuhrmann, N.S.M. Geohagen, C.L. Gnehm, L.A. McDonald, K.V. Small, C.M. Fraser, H.O. Smith, J.C. Venter, Science 269 (1995) 496.
[3] D.C. Webb, A.W. Cripps, J. Med. Microbiol. 47 (1998) 1059.
[4] T.M. Wizemann, J.E. Adamou, S. Langermann, Emerg. Infect. Dis. 5 (1999) 39.
[5] J.M. Musser, S.J. Berenkamp, D.M. Granoff, R.K. Selander, Infect. Immun. 52 (1986) 183.
[6] C.L. Nilsson, Am. J. Pharmacogenom. 2 (2002) 59.
[7] O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[8] M. Fountoulakis, B. Takacs, H. Langen, Electrophoresis 19 (1998) 761.
[9] A.J. Link, L.G. Hays, E.B. Carmack, J.R. Yates III, Electrophoresis 18 (1997) 1314.
[10] H. Langen, B. Takacs, S. Evers, P. Berndt, H.W. Lahm, B. Wipf, C. Gray, M. Fountoulakis, Electrophoresis 21 (2000) 411.
[11] C.L. Nilsson, P. Davidsson, Mass Spectrom. Rev. 19 (2000) 390.
[12] V. Santoni, M. Molloy, T. Rabilloud, Electrophoresis 21 (2000) 1054.
[13] B. Herbert, Electrophoresis 20 (1999) 660.

[14] B.R. Herbert, M.P. Molloy, A.A. Gooley, B.J. Walsh, W.G. Bryson, K.L. Williams, Electrophoresis 19 (1998) 845.
[15] M.P. Molloy, Anal. Biochem. 280 (2000) 1.
[16] M.P. Molloy, B.R. Herbert, B.J. Walsh, M.I. Tyler, M. Traini, J.C. Sanchez, D.F. Hochstrasser, K.L. Williams, A.A. Gooley, Electrophoresis 19 (1998) 837.
[17] C.L. Nilsson, T. Larsson, E. Gustafsson, K.-A. Karlsson, P. Davidsson, Anal. Chem. 72 (2000) 2148.
[18] E. Gustafsson, K. Thorén, T. Larsson, P. Davidsson, K.-A. Karlsson, C.L. Nilsson, Rapid Commun. Mass Spectrom. 15 (2000) 428.
[19] P. Davidsson, C.L. Nilsson, Biochim. Biophys. Acta 1473 (1999) 391.
[20] W. El-Adhami, J.M. Kyd, D.A. Bastin, A.W. Cripps, Infect. Immun. 67 (1999) 1935.
[21] T.J. Reilly, D.L. Chance, A.L. Smith, J. Bacteriol. 181 (1999) 6797.
[22] D.N. Chakravarti, M.J. Fiske, L.D. Fletcher, R.J. Zagursky, Vaccine 19 (2001) 601.
[23] P. Cash, Electrophoresis 21 (2000) 1187.