In vivo expression of Neisseria meningitidis proteins homologous to the Haemophilus influenzae Hap and Hia autotransporters

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Abstract

The genome sequences of Neisseria meningitidis serogroup B strain MC58 and serogroup A strain Z2491 were systematically searched for open reading frames (ORFs) encoding autotransporters. Eight ORFs were identified, six of which were present in both genomes, whereas two were specific for MC58. Among the identified ORFs was the gene encoding the known autotransporter IgA1 protease. The deduced amino acid sequences of the other identified ORFs were homologous to known autotransporters and found to contain an N-terminal signal sequence and a C-terminal domain that could constitute a β-barrel in the outer membrane. The ORFs NMB1985 and NMB0992, encoding homologs of the Hap (for Haemophilus adhesion and penetration protein) and Hia (for Haemophilus influenzae adherence protein) autotransporters of H. influenzae, were cloned from serogroup B strain H44/76 and expressed in Escherichia coli. Western blots revealed that all sera of patients (n=14) and healthy carriers (n=3) tested contained antibodies against at least one of the recombinant proteins. These results indicate that both genes are widely distributed among N. meningitidis isolates and expressed during colonization and infection. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Autotransporter; In vivo expression; Outer membrane protein; Adhesin; Neisseria meningitidis

1. Introduction

Encapsulated Neisseria meningitidis is a major cause of septicaemia and meningitis world-wide [1]. Most disease-causing isolates express one of the polysaccharide capsules of serogroups A, B, C, Y and W135. Effective vaccines, at least for adults, have been developed based upon the capsular polysaccharides of serogroups A, C, Y and W135 [2]. However, the serogroup B capsule is poorly immunogenic, since it mimics a structure present on the surface of cells of host tissues. Moreover, capsular polysaccharides elicit immune responses with a poor memory, and such vaccines are only effective in children over 2 years of age, whereas the disease is a major problem in younger children. Outer membrane (OM) vesicles containing the major outer membrane proteins (OMPs) have been evaluated as an alternative vaccine [3,4]. These major OMPs, however, are antigenically diverse and their expression is subject to phase variation. Therefore, these vesicle vaccines provided only limited protection [3,5].

Vaccines may improve by the inclusion of minor OMPs, provided that these OMPs are expressed in sufficient quantities to elicit an immune response and that the resulting antibodies are cross-reactive to a broad range of strains. In addition, such a vaccine component should be expressed either during colonization of the epithelial layers of the upper respiratory tract or during infection of the blood and the meninges. The search for such additional components is greatly facilitated by the recent availability of the genome sequences of two N. meningitidis strains [6,7].

In this study, the genome sequences were searched for genes putatively encoding autotransporters. Autotransporters comprise a family of secreted proteins able to translocate themselves over the OM (hence their name) [8]. Their primary sequence harbors an N-terminal signal sequence directing transport over the inner membrane by
the Sec system and a C-terminal domain of approximately 30 kDa presumably forming a β-barrel pore in the OM for translocation of the functional domain across the membrane. After translocation, the active domain is cell-surface exposed or secreted into the medium. Their surface exposure renders these types of proteins attractive candidates for application in a vaccine. Moreover, the majority of autotransporters studied to date were implicated in host–pathogen interactions [9], and Pertactin, an autotransporter of Bordetella pertussis was included in an acellular vaccine currently in use [10].

The first autotransporter described was the IgA1 protease of Neisseria gonorrhoeae [11], homologs of which were also found to be ubiquitous in N. meningitidis isolates [12]. Until recently, information on other genes encoding autotransporters in Neisseria species was limited [13–15]. Here, we report the identification of seven N. meningitidis genes, apart from iga encoding IgA1 protease, putatively encoding autotransporters. Two of them, homologous to autotransporters of Haemophilus influenzae, were cloned. We show that these proteins are expressed in vivo, since they are recognized by antibodies in sera from patients convalescent from meningitis and in sera from healthy carriers.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

N. meningitidis strain H44/76 (B:15:P1.7,16) was originally isolated from the cerebrospinal fluid of a meningitis patient in Norway and was shown to belong to the ET-5 group of strains associated with many cases of disease [16]. Cultures of the strain were started from frozen stocks and grown overnight in Tryptic soy broth. Escherichia coli strains used, DH5α, Top10F’ (Invitrogen, Groningen, The Netherlands) and BL21(DE3) (Novagen, Madison, WI, USA), were grown on Luria broth (LB) supplemented with 100 μg ml−1 ampicillin for plasmid maintenance and with 0.5% glucose for full repression of the lac promoter. Cloning and expression vectors used were pCRII-topo (Invitrogen), pET11a and pET16b (Novagen).

2.2. Database searches

The genome sequences of N. meningitidis serogroup A strain Z2491 [6] and serogroup B strain MC58 [7] were searched for genes putatively encoding autotransporters by homology searching using the sequences of 28 autotransporters from 10 different bacterial species (Table 1). This list was based upon the sequences described by [16] and [10], and, additionally, TibA [17], PssA [18] and Vag8 [19] were included. Database searches were performed using the web sites of The Sanger Centre (http://www.sanger.ac.uk/projects/n_meningitidis/blast_server; this site was used extensively in the period prior to publication of the two genome sequences) and the NCBI Blast and the NCBI Blast of Finished and Unfinished Microbial Genomes web pages (via http://www.ncbi.nlm.nih.gov/blast). The genomes were searched with the tBlastn program [20], comparing the amino acid sequences of the autotransporters to the genomes translated into six reading frames. Default search matrices were used, but filters for regions of low complexity were switched off. Hits that showed more than 20% similarity over regions larger than 250 amino acid residues were further investigated. The complete ORFs corresponding to the homologous regions were retrieved and compared to sequences in the GenBank database to identify any homologous N. meningitidis sequences previously submitted. The selected
ORFs were also searched for the presence of an N-terminal signal sequence, using the signalP program (http://www.cbs.dtu.dk/services/signalp-2.0; [21]). However, the N-terminal 100 instead of the recommended 70 amino acid residues were used, since autotransporters tend to have long signal sequences [9]. The C-terminal 350 amino acid residues were analyzed by hand for the presence of a domain that could form a β-barrel structure [8]. Finally, the last nine amino acid residues were compared to the signature sequence for the postulated last β-strand of the β-barrel, i.e. [LIVFGASY]-[NQHEGAFITS]-[LIVA]-[NGSKT]-[LIVMFYG]-[RKQSTGN]-[LIFVYW]-[STA-KRNVQEM]-[FW] [9], whereby the last three residues were described as the most important [22]. DNA and protein sequence analysis and handling were done using the CloneManager 5.0 software package (Scientific and Educational Software, Durham, NC, USA). Additional sequence alignments were performed using the facilities of the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu) and the EBI server (http://www.ebi.ac.uk/tools). Comparison to the unfinished N. gonorrhoeae FA1090 genome sequence was done at http://www.genome.ou.edu/gono.

2.3. Cloning of ORFs NMB0992 and NMB1985

Primers NMB0992Start (ggatccgagatatccggcatcatttga), NMB0992End (caacacctcataagcagaacgtgga), NMB1985Start (catgacctgaaacaaccgaacaggacacgga) and NMB1985End (gaagattcagcctgaaccttttagt) were designed to amplify the ORFs of NMB0992 and NMB1985 from the start codon up to and including the stop codon, without any up- or downstream sequences. They were based upon the Z2491 sequences (available at the time of primer selection), but all showed 100% identity to the comparable MCS8 regions at the DNA level. Restriction sites (underlined) were included in the primers to facilitate cloning. In primer NMB0992Start, an NdeI site was included, whereas an NcoI site was included in primer NMB1985Start. In the latter case, this manipulation resulted in the substitution of the second amino acid of the ORF (E for K), which, however, was not expected to disrupt signal sequence function (not shown). In both cases, the primer located at the end of the gene included a BglII site.

PCR reactions were performed in a Biometra PCR machine, using Pwo DNA polymerase (Boehringer Mannheim, Germany) for NMB0992, and the High Fidelity Long Range PCR kit (Boehringer Mannheim) for NMB1985. Reactions were performed in the presence of 1% dimethylsulfoxide, according to the manufacturer’s prescription, using genomic DNA of N. meningitidis strain H44/76 as the template. This DNA was purified using the Qiagen Genomic DNA Midi preps (Qiagen, Hilden, Germany) according to manufacturer’s prescription. PCR reactions typically included 30 cycles of amplification, and the annealing temperature ranged from 55 to 62°C. After cycling, the reaction products were incubated for 30 min at 72°C in the presence of Taq DNA polymerase (MBI Fermentas, St. Leon-Roth, Germany) and fresh nucleotides to extend the products with adenine nucleotides to facilitate TA-cloning (Invitrogen). PCR fragments of the expected length were isolated from gel using the Qiaquick gel isolation kit (Qiagen) and cloned into pCRII-topo (Invitrogen), using the methods described by the manufacturer.

The ORF of NMB0992 was cut from the pCRII-topo vector by restriction with NdeI and BamHI, and cloned into expression vector pET11a restricted with the same enzymes, resulting in plasmid pPU100. The ORF was found to contain an extra BglII site within the coding region, requiring the use of the BamHI site located in the multiple cloning region of pCRII-topo. The ORF of NMB1985 was cut from the pCRII-topo clone by restriction with NcoI and BglII and cloned into expression vector pET16b restricted with NcoI and BamHI, resulting in plasmid pPU200. The plasmids pPU100 and pPU200 contained the ORF under the control of the T7 promoter [23] and were checked by restriction enzyme digestion and sequencing of the fusion sites using universal T7 primers hybridizing to regions up- and downstream of the insertion site.

The ORF of NMB1985 was completely sequenced using the pCRII-topo plasmid containing the PCR product as a template. Primers used were the universal M13 primers and a collection of 13 primers based upon the sequence of NMB1985 of MC58 and the resulting sequences completely covered the ORF on both strands of the DNA. All sequencing reactions were done with the BigDye sequencing kit (Perkin Elmer Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands) and on an ABI310 sequencer (Perkin Elmer).

2.4. Overproduction of proteins encoded by NMB0992 and NMB1985 in E. coli

E. coli strain BL21(DE3), containing an inducible T7 RNA polymerase gene (Novagen), was transformed with plasmids pET16b, pPU100 and pPU200. Fresh overnight cultures were diluted 1:100 in LB with 0.5% glucose and grown to an OD600 of 0.6. Expression of the genes under the control of the T7 promoter was then induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, after which incubation was prolonged for another 2 h.

2.5. Human sera and other antibodies

Sera of 10 patients convalescent of meningococcal disease, three healthy carriers of N. meningitidis, and three healthy non-carriers were obtained from the collection of the Department of Medical Microbiology, Academic Medical Centre, Amsterdam, The Netherlands. The infecting strains of the patients and the colonizing strains of the
carriers have been serotyped. Four sera of convalescent patients and one non-carrier serum were obtained from the collection of the National Institute of Health and the Environment (RIVM), Bilthoven, The Netherlands. Antibodies against *H. influenzae* Hia (for *H. influenzae* adherence protein) and Hap (for *Haemophilus* adhesion and penetration protein) were kindly donated by Dr. St. Gme (Washington University School of Medicine, St. Louis, MO, USA).

2.6. SDS–PAGE and Western blotting

Proteins were analyzed by SDS–PAGE with an 8% (w/v) polyacrylamide running gel and visualized by staining with Coomassie brilliant blue. Whole cell lysates of equal amounts of cells were loaded.

To detect binding of antibodies to the overproduced proteins, Western blots were made containing proteins from isolated membrane fractions. To obtain the membrane fraction, cells were harvested by centrifugation at 7000×*g* and resuspended in a buffer of 50 mM Tris–HCl, pH 8.0, and 2 mM EDTA, and then disrupted in batches of 20 ml by sonication for 2×15 s in a Branson sonifier at full power while on ice and with 15 s in between steps. Lysates were cleared from unbroken cells and aggregates by two subsequent centrifugation steps at 14000×*g* for 20 min. The membrane fractions (mostly OMs) were pelleted by 5 min centrifugation at 100 000×*g*. The resulting pellets were dissolved in 5 mM Tris–HCl, pH 7.6. Samples of the fractions containing equal amounts of proteins, as derived from the OD_{280} of the preparations, were separated by SDS–PAGE and blotted upon 0.45 μm Protran filters (Schleicher and Schuell, Dassel, Germany) using the Protean II minigel blotting system (Bio-Rad Laboratories, Veenendaal, The Netherlands) at 100 V for 1 h. Unspecific binding of antibodies to filters was prevented by overnight incubation in phosphate-buffered saline (PBS), pH 7.0, supplemented with 0.5% Protifar (Nutricia, Zoetermeer, The Netherlands) and 0.1% Tween 20 (Merck and Co., NJ, USA). The sera were diluted 1:500 or 1:750 in the same buffer and applied for 1 h to the blots. After extensive washing, the blots were incubated with goat anti-human IgG conjugated to horseradish peroxidase (Dako, Glostrup, Denmark) at a dilution of 1:5000 in the same buffer. Binding of antibodies was visualized by chemiluminescence using the ECL kit (Pierce, Rockford, IL, USA).

2.7. Protease-accessibility assay

*E. coli* BL21(DE3) cells producing recombinant proteins were harvested by centrifugation at 7000×*g*. Pellets were washed in PBS, pH 7.6, and resuspended in 10 mM Tris–HCl, pH 7.6, 10 mM MgCl₂ to an OD_{600} of 1.0 and put on ice for 10 min. Next, 500-μl samples of the cell suspensions were incubated with different amounts of proteinase K or trypsin on ice for 20 min. Protease digestion was stopped by adding 10 μl of a 100 mM phenylmethylsulfonyl fluoride solution in isopropanol, followed by an incubation on ice for 30 min. Cells were harvested by centrifugation and resuspended in 50 μl of sample buffer and proteins were separated by SDS–PAGE. To test whether the protease was active on the intra-cellular proteins, cells were resuspended in a buffer containing 10 mM Tris–HCl, pH 7.6, 5 mM EDTA resulting in the disintegration of the OM.

2.8. Immunofluorescence microscopy

*E. coli* BL21(DE3) cells producing recombinant proteins were harvested by centrifugation. Pellets were washed and resuspended in PBS to an OD_{600} of 1.0. 300 μl cell suspension was incubated for 30 min with 14-mm cover slips that had been coated for 30 min with poly-l-lysine (Sigma, St. Louis, MO, USA). Cover slips with bound cells were washed twice with PBS and fixed overnight with 2% formaldehyde in PBS at 4°C. Next, cover slips were washed twice with PBS and then incubated for 1 h at room temperature with PBS supplemented with 0.05% Tween 20 (Sigma) and 3% bovine serum albumin (BSA) (Sigma). Serum 780646 was diluted 1:250 in the same buffer and then pre-incubated for 1 h with IPTG-induced *E. coli* cells containing pET16b, with the amount of cells being equivalent to a 1-ml culture with an OD_{600} of 1.0. Cells were removed by centrifugation and the pre-adsorbed serum was incubated with the cover slips for 1 h at room temperature. The cover slips were washed thrice with PBS with 0.05% Tween 20 and subsequently incubated for 1 h with goat anti-human IgG antibodies to which the fluorogenic dye Alexa was coupled (Molecular Probes, Leiden, The Netherlands), diluted 1:300 in PBS with 0.05% Tween 20 and 3% BSA. After washing thrice with PBS with 0.05% Tween 20, cells were fixed again with 2% formaldehyde in PBS and washed, after which they were mounted on glass slides. The results were assessed using a fluorescence microscope. As a control, cover slips with cells were incubated with rabbit antibodies against β-lactamase at a dilution of 1:3000 in the same buffer as used for the human serum. In these cases, the second antibody was Alexa-conjugated goat anti-rabbit IgG antiserum. When applicable, the OMs of cells were permeabilized after the first fixation step by incubating the cover slips for 20 min in PBS with 0.5% Triton X-100 at room temperature, after which the cover slips were washed twice with PBS.

3. Results

3.1. Identification of ORFs putatively encoding autotransporters

The genome sequences of *N. meningitidis* serogroup A strain Z2491 and serogroup B strain MC58 were screened...
for the presence of ORFs putatively encoding proteins with homology to the amino acid sequences of 28 known autotransporters (Table 1). Initial screening led to the identification of 14 ORFs, showing homology to one or more autotransporters. Two of them, NMB0545 and NMB0711 and their serogroup A counterparts NMA0724 and NMA0916, were discarded because a signal sequence could not be identified. One ORF, NMB1540/NMA1739, was discarded because it showed over 80% sequence identity to LbpA, a well-known integral OMP of N. meningitidis [24]. One ORF, NMA0688, which showed homology to the filamentous hemagglutinin of B. pertussis was discarded since its C-terminal sequence did not match the C-terminal signature sequence of autotransporters. Moreover, an accessory protein involved in OM localization of filamentous hemagglutinin has been described [25], and a homolog of this protein was found adjacent to NMA0688. Interestingly, NMA0688 was only present in the serogroup A sequence. Finally, two ORFs, NMB0497 and NMB01779, only present in the serogroup B sequence and encoding proteins homologous to hemolysins, also had C-terminal sequences differing from the signature sequence and were subsequently discarded. The remaining eight ORFs (Table 2) complied with the criteria set: they encoded polypeptides homologous to known autotransporters, had an N-terminal signal sequence and a putative β-barrel domain containing the signature sequence of autotransporters at their C-terminus. The two genomes were also screened with the C-terminal signature sequence as a probe [9], but this approach did not lead to the identification of additional putative autotransporters.

Six of the eight ORFs were identified in both MC58 and Z2491, with homologies between the corresponding ORFs ranging from 85 to 98% sequence identity at the amino acid level (Table 2). The highest identity was found between the ORFs encoding the IgA1 protease [12]. Both were 73% identical to the IgA1 protease of N. gonorrhoeae, the first autotransporter described in detail [11]. ORFs homologous to NMB1994 and NMB1998 were not identified in the serogroup A sequence. However, these ORFs are not located in the three DNA regions specific for MC58 [7].

The presence of homologs of the identified N. meningitidis ORFs in the unfinished N. gonorrhoeae genome sequence was examined using both the nucleotide and deduced amino acid sequences for screening. In addition to iga encoding IgA1 protease, homologs of NMB1525 (72% identity at the protein level) and NMB1985 (95% identity) were identified. A nucleotide sequence homologous to NMB0312 was found, but the ORF was disrupted by premature stop codons. Similarly, a region homologous to NMB1969 was found, but the coding region appeared to be split up into two separate ORFs. No homologs were found of ORFs NMB0992, NMB1994 and NMB1998.

3.2. Cloning of ORFs NMB0992 and NMB1985 from vaccine strain H44/76

For the cloning of ORFs, serogroup B strain H44/76 was chosen since it has been used for vaccine development [3,4]. The ORFs NMB0992 and NMB1985 were selected as first targets for cloning, since they encode proteins homologous to the Hia and Hap proteins of H. influenzae, respectively. Like N. meningitidis, H. influenzae colonizes the upper respiratory tract upon entry of the human body, and the Hia and the Hap proteins were both suggested to play a role in the colonization of epithelial cell layers [26,27]. Furthermore, phase-variable expression of both ORFs seemed unlikely because of the complete absence (NMB0992) or limited number (NMB1985) of repeated nucleotide sequences [28].

DNA fragments containing the ORFs were amplified from genomic DNA of H44/76 by PCR and amplicons of expected lengths were purified from the gel and cloned. Their identity was confirmed by sequencing. The NMB1985 ORF was completely sequenced. It differed in

Table 2

<p>| Identified ORFs in the N. meningitidis genomes with homology to known autotransporters |
|-----------------------------------------|----------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Identifying ORFs</th>
<th>Homologous autotransporter</th>
<th>Signal sequence</th>
<th>C-terminal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC58</td>
<td>Z2491 (% identity)</td>
<td>name, function, species (% identity/% similarity)</td>
<td></td>
</tr>
<tr>
<td>NMB0312</td>
<td>NMA2175 (96)</td>
<td>Aida-L, adhesin, E. coli (32/49)</td>
<td>41</td>
</tr>
<tr>
<td>NMB1525</td>
<td>NMA1725 (85)</td>
<td>Aida-L, adhesin, E. coli (30/46)</td>
<td>34</td>
</tr>
<tr>
<td>NMB0700</td>
<td>NMA0005 (98)</td>
<td>IgA1 protease, N. gonorrhoeae (73/75)</td>
<td>20</td>
</tr>
<tr>
<td>NMB0992</td>
<td>NMA1200 (86)</td>
<td>Hia, adhesin, H. influenzae (52/66)</td>
<td>51</td>
</tr>
<tr>
<td>NMB1969</td>
<td>NMA0478 (96)</td>
<td>Sop-h2,unknown, S. marcescens (25/39)</td>
<td>27</td>
</tr>
<tr>
<td>NMB1985</td>
<td>NMA0457 (89)</td>
<td>Hap, adhesin/invasin, N. meningitidis (56/65)</td>
<td>42</td>
</tr>
<tr>
<td>NMB1994</td>
<td>–</td>
<td>UspA2, serum resistance, M. catarrhalis (39/59)</td>
<td>25</td>
</tr>
<tr>
<td>NMB1998</td>
<td>–</td>
<td>Tsh, hemagglutinin, E. coli (30/45)</td>
<td>26</td>
</tr>
</tbody>
</table>

*The values and sequences given apply to the ORFs as identified in the genome sequence of serogroup B strain MC58.

1Length in amino acid residues based upon signal sequence predictions [21].

2Residues not in accordance with the signature sequence [LIVFGASY]-[NQHEGAIFTS]-[LIVA]-[NGSKRT]-[LIVMFYG]-[RKQSTGNN]-[STAKRNVQEM]-[FW] for the C-terminus of autotransporters [9] are underlined.

3The % identity and similarity given refers to the derived amino acid sequences of the ORFs.
only two nucleotides from NMB1985 of MC58. The first substitution, at position 4, resulted from the cloning procedure. The second substitution, at position 3229, resulted in a lysine residue at position 1077 of the amino acid sequence, where there is a glutamate in NMB1985 of MC58. NMB0992 of strain H44/76 was previously sequenced by others (accession number AF226374 [15]). The deduced amino acid sequence of the ORF is 100% identical to that of MC58.

The ORFs of NMB0992 and NMB1985 were cloned into expression vectors resulting in plasmids pPU100 and pPU200, respectively (see Section 2). Expression of the genes in E. coli BL21(DE3) led to the production of recombinant proteins (Fig. 1). The precursor and mature forms of the Hia-homolog NMB0992 have calculated molecular masses (MWs) of 62.1 and 56.5 kDa, respectively, and a protein with an apparent molecular mass (Mr) corresponding to the precursor was produced (Fig. 1, lane 2). Additional bands with a much higher Mr also appeared on the gel (Fig. 1, lane 2). These probably represented multimeric complexes of the protein. The precursor and processed forms of the Hap-homolog NMB1985 have calculated MWs of 160 kDa and 155 kDa, respectively, and a band with approximately this Mr was observed on the gel (Fig. 1, lane 3). Attempts to determine the N-terminal sequence of the major protein bands were unsuccessful, most probably because the N-termini were blocked. This suggested that in both cases, the majority of the proteins produced corresponded to the unprocessed precursor forms.

The Hap protein of H. influenzae was reported to release its functional domain into the medium by autoproteolytic cleavage [27]. Such release was not observed for the Hia protein [29]. To investigate whether the neisserial homologs are proteolytically cleaved in E. coli, medium of E. coli cells expressing the proteins was concentrated by TCA precipitation and analyzed by SDS-PAGE. In neither case was a secreted product detected in the medium.

3.3. Immunogenicity and in vivo expression of NMB0992 and NMB1985

To investigate whether the Hia- and Hap-homologs NMB0992 and NMB1985 are immunogenic in humans, a collection of sera was tested in Western blot experiments for the presence of antibodies recognizing the recombinant proteins (see Table 3). Twelve of a panel of 14 sera of convalescent patients tested recognized both NMB0992 and NMB1985 of strain H44/76, when produced in E. coli (see serum 790245 in Fig. 2A for an example). Serum 790269 only recognized NMB1985 (Fig. 2B), whereas serum 780287 only recognized NMB0992 (Fig. 2C). Of two patients, serum taken at the day of hospital admittance and thus early in disease, could be tested as well. These sera showed a weaker reaction (Fig. 2, panels A and D), as compared with the convalescent sera. Moreover, in the case of serum 790767, antibodies recognizing NMB1985 were only present at convalescence (Fig. 2D). All sera of healthy carriers tested (n = 3) contained antibodies recognizing both proteins (Fig. 3A–C), although the responses appeared lower than those of patient sera (Table 3). Of one healthy carrier, two sera were available (Fig. 3C), taken at 9-month interval, during which two different N. meningitidis strains colonized this person (Table 3). The sera contained antibodies to both NMB0992 and NMB1985, but the responses to NMB0992 decreased over time, whereas the response to NMB1985 was somewhat boosted, possibly as a result of the colonization by the second strain. Three of the four sera of non-carriers did not contain antibodies recognizing either protein (Fig. 3D–F), whereas one recognized NMB1985 clearly and NMB0992 faintly (Fig. 3G). Apparently, the person involved had been a carrier prior to the blood sampling. Taken together, the results demonstrate that the Hia- and Hap-homologs NMB0992 and NMB1985 were expressed by a wide range of N. meningitidis strains during infection and colonization and that these proteins are immunogenic in humans.
3.4. Cellular localization of the recombinant proteins in E. coli

To determine whether the proteins encoded by NMB0992 and NMB1985 were targeted to the OM of E. coli with the functional domains exposed on the bacterial cell surface, two approaches were followed. In the first approach, protease accessibility of the recombinant proteins in whole cells was assessed. Trypsin (Fig. 4A) and proteinase K (Fig. 4B) treatment of whole cells expressing the two recombinant proteins revealed that the higher molecular mass bands of NMB0992 were highly sensitive to these proteases, since the bands were digested at low protease concentrations, at which apparently no other bands were affected (Fig. 4A, lane 4 and Fig. 4B, lane 5). This would suggest that the putative multimeric forms of NMB0992 were accessible for protease digestion at the cell surface. The monomeric bands of NMB0992 and NMB1985 appeared less protease sensitive, suggesting that these proteins are either not surface exposed, or are intrinsically resistant to proteases.

In the second approach, serum 780646, which contained antibodies recognizing both NMB0992 and NMB1985, was used in immunofluorescence microscopy. This serum was selected on the basis of its limited cross-reaction with E. coli bands in Western blot, and background binding to E. coli proteins was further diminished by pre-adsorption of the serum with E. coli cells containing pET16b. Next, it was incubated with E. coli cells producing either recombinant NMB0992 or NMB1985, or containing the empty vector. Control cells, containing the empty vector, were hardly stained by the serum (Fig. 5A). The serum very clearly labelled cells expressing recombinant NMB1985, the Hap homolog (Fig. 5C). Immunostaining of cells expressing the Hia homolog was less clear (Fig. 5B), but staining was above the level of that of the controls. The integrity of the cells was tested by incubation with anti-

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Table 3
Presence of antibodies against the recombinant proteins NMB0992 and NMB1985 in human sera

<table>
<thead>
<tr>
<th>Donor</th>
<th>serum number</th>
<th>gender</th>
<th>age (years)</th>
<th>Source*</th>
<th>N. meningitidis isolate</th>
<th>Recognition of recombinant proteinsb</th>
<th>NMB0992 (Hia homolog)</th>
<th>NMB1985 (Hap homolog)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meningitis patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>780287</td>
<td>male</td>
<td>6</td>
<td>AMC</td>
<td>B:2b:P1.5,2</td>
<td>++</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>780613</td>
<td>male</td>
<td>11</td>
<td>AMC</td>
<td>B:4:P1.15</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>780645</td>
<td>male</td>
<td>17</td>
<td>AMC</td>
<td>C:4:P1.15</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>780646</td>
<td>female</td>
<td>9</td>
<td>AMC</td>
<td>A:NT:P1.6</td>
<td>++</td>
<td>++</td>
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<tr>
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<td>NG:4:P1.5,2</td>
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</table>


**++: good recognition; +: detectable recognition; –: no recognition

*a: not known

*b: serum taken at hospital admittance; conv: serum taken at convalescence

cThis person was carrier of an unencapsulated variant of serogroup B strain H44/76

dcarrier of C:2a:P1.5,2 for 6 months until July 1998

eThese persons were non-carriers at the time of blood sampling, as judged from throat swabs. At least for one person (NC1), previous carriership is known.

3.4. Cellular localization of the recombinant proteins in E. coli

To determine whether the proteins encoded by NMB0992 and NMB1985 were targeted to the OM of E. coli with the functional domains exposed on the bacterial cell surface, two approaches were followed. In the first approach, protease accessibility of the recombinant proteins in whole cells was assessed. Trypsin (Fig. 4A) and proteinase K (Fig. 4B) treatment of whole cells expressing the two recombinant proteins revealed that the higher molecular mass bands of NMB0992 were highly sensitive to these proteases, since the bands were digested at low protease concentrations, at which apparently no other bands were affected (Fig. 4A, lane 4 and Fig. 4B, lane 5). This would suggest that the putative multimeric forms of NMB0992 were accessible for protease digestion at the cell surface. The monomeric bands of NMB0992 and NMB1985 appeared less protease sensitive, suggesting that these proteins are either not surface exposed, or are intrinsically resistant to proteases.

In the second approach, serum 780646, which contained antibodies recognizing both NMB0992 and NMB1985, was used in immunofluorescence microscopy. This serum was selected on the basis of its limited cross-reaction with E. coli bands in Western blot, and background binding to E. coli proteins was further diminished by pre-adsorption of the serum with E. coli cells containing pET16b. Next, it was incubated with E. coli cells producing either recombinant NMB0992 or NMB1985, or containing the empty vector. Control cells, containing the empty vector, were hardly stained by the serum (Fig. 5A). The serum very clearly labelled cells expressing recombinant NMB1985, the Hap homolog (Fig. 5C). Immunostaining of cells expressing the Hia homolog was less clear (Fig. 5B), but staining was above the level of that of the controls. The integrity of the cells was tested by incubation with anti-
be involved in host–pathogen interactions [30]. A systematic search of the recently published genome sequences of two N. meningitidis strains revealed the presence of eight ORFs putatively encoding autotransporters in the genome of serogroup B strain MC58, six of which were also present in that of serogroup A strain Z2491. The different repertoire of autotransporter-encoding genes in the two genomes suggests that additional autotransporters might be identified when more disease isolates are screened. Such additional autotransporters might be less interesting for vaccine development because of lack of conservation. The genome of N. gonorrhoeae strain FA1090 contained only three intact homologs of the N. meningitidis autotransporters. Two disrupted homologs were identified, which might be intact in other isolates.

Some of the ORFs identified have appeared in the literature or the GenBank database, apart from their presence in the sequenced genomes. The iga of N. meningitidis isolates have been studied extensively [12]. The nucleotide sequences of NMB0312 and NMB1525 have been identified by the presence of sequence repeats [13], indicative of phase-variable expression. A second, unpublished entry was found for NMB0312 (accession number AF118122) and during the preparation of this paper, NMB0312 (designated AutA) was reported to encode a CD4+ T-cell and B-cell stimulatory antigen [31]. The authors also identified

4. Discussion

The class of autotransporter proteins might include interesting vaccine constituents since most of them appear to

Fig. 2. Western blots of membrane preparations of E. coli BL21(DE3) strains expressing recombinant proteins NMB0992 and NMB1985 from pPU100 and pPU200, respectively. Blots were incubated with the patient sera indicated underneath the panels, taken either at hospital admittance or at convalescence, as indicated. Bands corresponding to NMB0992 and NMB1985 are labelled with asterisks and dots, respectively.

serum against the periplasmic protein β-lactamase. This serum stained some of the cells expressing the recombinant proteins, but the level of labelling was lower than with the patient serum (Fig. 5D–F). The β-lactamase antiserum could effectively stain cells when the OM was disrupted with Triton X-100 (results not shown). These results indicate that antigenic domains of the recombinant proteins were exposed on the cell surface.

Fig. 3. Western blots of membrane preparations of E. coli BL21(DE3) strains expressing recombinant proteins NMB0992 and NMB1985 from pPU100 and pPU200, respectively. Blots were incubated with the sera of healthy carriers (panels A–C), or non-carriers (panels D–F). Panel C shows two blots incubated with the sera of a carrier taken at a 9-month interval, as indicated. Bands corresponding to NMB0992 and NMB1985 are labelled with asterisks and dots, respectively.
NMB1525 (designated AutB) as a homolog of NMB0312 and demonstrated that antibodies against this protein failed to react with *N. meningitidis* grown in vitro. NMB1969 and NMB1985 were very recently entered in the database (accession numbers AJ277537 and AJ242535) without published account. Finally, NMB0992 (designated NhhA [1]) was identified as a putative vaccine candidate based upon its homology to Hia of *H. influenzae* [14] and in a genome-wide screen for antigens [15]. No database entries were found for NMB1994 and NMB1998.

In a first study of the vaccine potential of *N. meningitidis* autotransporters, we focused on two ORFs, NMB0992 and NMB1985, because they encode proteins that are very homologous to the Hia [26] and Hap [32] proteins of *H. influenzae*. Moreover, *N. meningitidis* and *H. influenzae* both colonize the upper respiratory tract, and it was suggested that the proteins of *H. influenzae* might play a role in that process by functioning as adhesins [26,27].

Expression of NMB0992 of serogroup B strain H44/76 in *E. coli* yielded a protein with an $M_r$ of 62 kDa, most probably representing the unprocessed full-length precursor protein, and two bands with $M_r$s of over 200 kDa. These higher $M_r$s bands might represent multimeric complexes, which appear to be surface-exposed, as suggested by the protease-accessibility experiments. Consistently, the immunofluorescence experiments indicated that at least a proportion of the recombinant protein produced was cell-surface-exposed.

The Hia homolog of strain MC58 was cloned in an independent study [14], and antibodies raised against this protein recognized a band with an $M_r$ of over 200 kDa. Higher $M_r$s complexes were not observed when Hia of *H. influenzae* was expressed in either *H. influenzae* or *E. coli* [29]. The Hia protein and its neisserial homolog differ considerably in size, being 1098 and 591 amino acid residues long, respectively. The C-terminal 318 residues of Hia fit with the model proposed for the autotransporter trans-
locator domain [8] since they putatively constitute a β-barrel of 14 amphipathic β-strands. In a sequence alignment, the translocator domain of NMB0992 appears to miss about 140 residues, which would result in the loss of about six to eight β-strands. The remaining number of β-strands would be too low for the formation of β-barrel with a pore of sufficient size to allow for the translocation of a polypeptide chain. Possibly, multimer formation could result in the formation of a β-barrel with a larger pore size. Such a multimeric organization would represent a new type of molecular organization for the autotransporter β-barrel domain, and would be reminiscent of the multimeric β-barrel formed in the OM by the TolC protein [33]. Like many of the autotransporters [9], the Hia protein of H. influenzae and its neisserial homologs were predicted to contain a long signal sequence of

![Image of immunofluorescence microscopy](image_url)

**Fig. 5.** Immunofluorescence microscopy of E. coli BL21(DE3) cells expressing recombinant proteins NMB0992 and NMB1985 from pPU100 and pPU200, respectively. Cells expressing recombinant proteins were incubated with patient serum 780646 or polyclonal rabbit serum recognizing periplasmic β-lactamase. Binding of antibodies was detected with anti-human or anti-rabbit IgG antibodies, respectively, both coupled to the fluorogenic dye Alexa. Cells were visualized by fluorescence microscopy. Panels A and D: background binding of serum to E. coli BL21(DE3) containing pET16b. Panels B and E: binding of serum to E. coli BL21(DE3) containing pPU100. Panel C and F: binding of serum to E. coli BL21(DE3) containing pPU200.
approximately 50 amino acid residues. When compared to normal signal sequences, they seemed extended at the N-terminus with aromatic and hydrophobic residues near the N-terminus and a signature sequence IAVSELAR preceding the charged N-domain [9].

The putative signal sequence of the Hap homologs of the Neisseriae are predicted to be 42 amino acid residues long, which is markedly longer than the 25 residues of the H. influenzae Hap signal sequence [22]. These signal sequences, however, do not have the characteristics of the long signal sequences of autotransporters. The neisserial Hap-like proteins showed the highest homology with Hap of H. influenzae in the functional domain (65% similarity), with a serine protease consensus motif located at the same position in the protein sequence. The C-terminal domains are less homologous (57% similarity). Thus, the functional domains of the proteins might share a similar function, most probably in adhesion to host cells [27], whereas the mode of translocation to the cell surface and the proteolytic cleavage of the proteins could be different. For H. influenzae, Hap autoproteolytic processing was observed upon expression in E. coli [22], which was not the case for NMB1985 of H44/76. This might indicate that autoproteolytic cleavage of the N. meningitidis protein does not occur. Alternatively, the majority of protein produced was not properly localized in E. coli and appeared in inclusion bodies. Nevertheless, immunofluorescence microscopy suggested that at least a proportion of the protein produced was exposed at the cell surface. A possibly important difference between the neisserial Hap homologs is that the NMA0457 protein of serogroup A strain Z2491 contains an RGD motif, which is a binding site for host proteoglycans in filamentous hemagglutinin of B. pertussis [34]. This motif is absent in the serogroup B Hap homologs and in the H. influenzae Hap.

Sera of convalescent patients, each infected by a different N. meningitidis isolate, contained antibodies that recognized either NMB0992 or NMB1985, or, in most cases, both proteins. Apparently, during infection both proteins were expressed by most of the N. meningitidis isolates. Moreover, limited antigenic variation is suggested, since antibodies in most tested patient sera recognized the recombinant proteins cloned from strain H44/76. The presence of genes encoding Hap-homologs in clinical isolates had not been studied to date, but is clearly demonstrated here. The gene encoding the Hia-homolog of MC58 was cloned in two independent studies [14,15] and both showed sequence variation of the Hia homologs in N. meningitidis. However, in both studies, antibodies raised against the recombinant protein appeared to be bactericidal and to recognize proteins from various strains. The data presented here add to this information the notion of expression during infection and the inter-strain cross-reactivity of human antibodies. In view of the reported sequence variation, this cross-reactivity may be somewhat surprising, but apparently the proteins share common epitopes that might be functionally important. Interestingly, we found no binding of antibodies raised against the C-terminal domains of the Hap and Hia proteins of H. influenzae to the recombinant N. meningitidis H44/76 proteins (results not shown).

Included in our tests were sera from healthy carriers that contained antibodies against both recombinant proteins, suggesting that the proteins were also expressed during asymptomatic colonization of the upper respiratory tract.

In conclusion, we have identified the repertoire of autotransporters present in the genomes of MC58 and Z249. Further characterization of two of these genes, encoding homologs of the Hia and Hap proteins of H. influenzae, showed that these proteins were most likely present during infection and colonization. Therefore, these proteins are interesting candidates to be included in a vaccine against N. meningitidis.

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References

[6] Parkhill, J., Achtman, M., James, K.D., Bentley, S.D., Churcher, C.,...


