A Functional Two-Partner Secretion System Contributes to Adhesion of *Neisseria meningitidis* to Epithelial Cells[†]

Corinna Schmitt,¹ David Turner,² Maria Boesl,¹ Marion Abele,¹ Matthias Frosch,¹ and Oliver Kurzai^{1*}

University of Wuerzburg, Institute of Hygiene and Microbiology, Josef-Schneider-Str. 2, E1, 97080 Wuerzburg, Germany,¹ and
Institute of Infections, Immunity and Inflammation, School of Molecular Medical Sciences, Centre for Biomolecular Sciences,
University Park, University of Nottingham, Nottingham NG7 2RD, United Kingdom²

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Neisseria meningitidis is a frequent commensal of the human nasopharynx causing severe invasive infections in rare cases. A functional two-partner secretion (TPS) system in *N. meningitidis*, composed of the secreted effector protein HrpA and its cognate transporter HrpB, is identified and characterized in this study. Although all meningococcal strains harbor at least one TPS system, the *hrpA* genes display significant C-terminal sequence variation. Meningococcal genes encoding the TPS effector proteins and their transporters are closely associated and transcribed into a single mRNA. HrpA proteins are translocated across the meningococcal outer membrane by their cognate transporters HrpB and mainly released into the environment. During this process, HrpA is proteolytically processed to a mature 180-kDa form. In contrast to other known TPS systems, immature HrpA proteins are stable in the absence of HrpB and accumulate within the bacterial cell. A small percentage of mature HrpA remains associated with the bacteria and contributes to the interaction of meningococci with epithelial cells.

Export of proteins to the surface and protein secretion are implicated in many aspects of bacterial life, including cell-to-cell communication, motility, and virulence. Highly sophisticated systems have evolved to ensure correct secretion of bacterial proteins, and many of them have been recognized as central virulence determinants. These include the type IV secretion system of *Helicobacter pylori* (2), type III secretion systems of members of the family *Enterobacteriaceae* (32), and bacterial autotransporters (18). Filamentous hemagglutinin (FHA) of *Bordetella pertussis* is the prototype of a family of large exoproteins in gram-negative bacteria that are secreted via the two-partner secretion (TPS) pathway (22, 23). FHA and related proteins, termed TpsA for two-partner secretion protein A, are translocated across the inner membrane via the universal Sec machinery (8). Transport across the outer membrane requires a specific transporter termed TpsB. In *B. pertussis*, this transporter is referred to as FhaC (23). The N-terminal region of the TpsA proteins contains a signal peptide for secretion via Sec and the adjacent TPS signal or secretion domain of approximately 245 amino acids (aa) that targets the TpsA protein to its cognate transporter, TpsB (8, 10, 22, 23). The genes encoding TpsA and TpsB are often found in close vicinity. Several TpsA proteins have been shown to contribute to virulence in plant and animal pathogens (5, 13, 38, 43, 48). *B. pertussis* FHA itself is both a major adhesion protein essential for establishing pathogen-host contact (29) and an important immunogen (26), representing the main constituent of the pertussis vaccine (35). By subtractive hybridization of DNA

regions specific for *Neisseria meningitidis* strain Z2491 and absent in *Neisseria gonorrhoeae*, Klee et al. were the first to describe a homologue of FHA in meningococci (27). All three available meningococcal genomes contain open reading frames (ORFs) encoding putative TPS systems (4, 34, 44) (Table 1). According to their homology with FHA, the corresponding genes have been annotated Hrp (for hemagglutinin/hemolysin-related proteins) in strain MC58 and will be referred to as HrpA (TpsA homologue) and HrpB (TpsB homologue), respectively. In contrast to the other strains, the genome sequence of serogroup B strain MC58 (44) contains two almost identical copies of the TPS system and three additional putative *hrpA* genes that differ mainly in their C-terminal sequences (Table 1) (46). In addition, another putative *hrpB* gene is present in strain MC58. The highest degree of sequence similarity between *B. pertussis* FhaB and meningococcal HrpA exists within the N-terminal region of the predicted proteins (22 to 25% identity) comprising the domain necessary for binding of FhaB to its transporter and secretion by the TPS pathway. In contrast, the C-terminal sequences of the meningococcal HrpA proteins show no relevant homology to *B. pertussis* FhaB.

N. meningitidis colonizes the upper respiratory tract of approximately 10% of healthy individuals (6, 7, 12) and only occasionally invades the bloodstream, causing life-threatening septicemia and meningitis (39). Whereas invasive meningococci belong to a few clonal lineages in the vast majority of cases, isolates from healthy carriers are genetically highly diverse (12). The genetic background of the variable virulence potential in *N. meningitidis* is still unclear. However, a thorough comparison of the genetic composition of hyperinvasive strains with carrier strains might lead to a better understanding of virulence in *N. meningitidis*.

By analyzing a large collection of meningococcal carrier isolates, we show that TPS systems are present in all *N.*

* Corresponding author. Mailing address: Institute of Hygiene and Microbiology, University of Wuerzburg, Josef-Schneider-Str. 2, E1, 97080 Wuerzburg, Germany. Phone: 49 (0)931 20146905. Fax: 49 (0)931 20146445. E-mail: okurzai@hygiene.uni-wuerzburg.de.

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TABLE 1. *hrpA* and *hrpB* genes in the sequenced meningococcal genomes

Strain	<i>hrpA</i>			<i>hrpB</i>		
	ORF ^a	Predicted protein length (aa)	% Identity (protein) to NMB1779	ORF	Predicted protein length (aa)	% Identity (protein) to NMB1780
Z2491	NMA0688	2,016	77.4	NMA0687	580	98.6
MC58	NMB1779	1,995	100	NMB1780	580	100
	NMB0497	1,976	93.5	NMB0496 ^b	559	99.3
	NMB0493	2,703	13.4	— ^c	—	—
	NMB1214	2,274	16.0	—	—	—
	NMB1768	2,514	13.9	NMB1762	595	44.5
FAM18	NMC0444	2,027	65.4	NMC0443	580	99.0

^a NMA, NMB, and NMC denote the genes in strain Z2491, MC58, and FAM18 genomes (44), respectively, encoding proteins homologous to *B. pertussis* FhaB (HrpA in meningococci) and FhaC (HrpB in meningococci).

^b The protein encoded by NMB0496 lacks the first 21 aa at the N terminus and thus presumably contains no functional signal sequence.

^c —, not detected; genome of no associated putative *hrpB* gene could be detected in the MC58 for NMB0493 and NMB1214.

meningitidis strains. However, the effector proteins display a uniquely high degree of sequence variation. By constructing deletion mutants, a function of HrpB as a transporter for HrpA and the contribution of HrpA to meningococcal adherence to host epithelial cells could be established.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 822 *N. meningitidis* isolates obtained from healthy carriers were used for analysis, which have been described previously (11, 12). Meningococcal strains used for infection experiments are listed in Table S1 in the supplemental material. Strains were stored in glycerol stock at -80°C and plated on gonococcal (GC) agar (BD Difco, Heidelberg, Germany) supplemented with Poly-ViteX (bioMérieux, Marcy l'Etoile, France). When appropriate, chloramphenicol (7 $\mu\text{g}/\text{ml}$ for *N. meningitidis* and 30 $\mu\text{g}/\text{ml}$ for *Escherichia coli*), spectinomycin (125 $\mu\text{g}/\text{ml}$ and 75 $\mu\text{g}/\text{ml}$, respectively), kanamycin (100 $\mu\text{g}/\text{ml}$ and 30 $\mu\text{g}/\text{ml}$, respectively), and/or erythromycin (7 $\mu\text{g}/\text{ml}$) were added to the medium for selection.

Molecular cloning, plasmids, and primers. Plasmids and primers are listed in Table S2 in the supplemental material. Recombinant DNA techniques were performed according to standard laboratory procedures. Plasmids were introduced into *E. coli* by chemical transformation. Meningococcal deletion mutants were constructed by integrating a selection marker into the gene of interest, simultaneously deleting part of the gene. Transformation of *N. meningitidis* was performed as described previously (15), and mutants were selected on appropriate media (see above). Detailed information regarding mutant construction can be found in the supplemental material. Meningococcal mutants used in this study are listed in Table S1 in the supplemental material. All mutant strains were checked for lipopolysaccharide (LPS) immunotype (L3 and L8) in a standard enzyme-linked immunosorbent assay using antibodies kindly provided by W. Zollinger and expression of major surface markers (Opc, Opa, and pili). Capsule expression was assayed using monoclonal antibodies MAb 735 (serogroup B) and MAb 924 (serogroup C) (14). MAb SM1 (anti-class 1 pili) was kindly provided by M. Virji (Bristol, United Kingdom), MAb AD211 (anti-class 2 pili), MAb B306 (anti-Opc), and MAb 4B12/C11 (anti-Opa) were kindly provided by Mark Achtman (Berlin, Germany).

Chromosomal DNA isolation, PCR, Southern blotting, and DNA sequencing. Chromosomal DNA was isolated from *N. meningitidis* using the Genomic-Tip system (QIAGEN, Hilden, Germany). Restriction enzymes and *Taq* polymerase were purchased from NEB (Frankfurt, Germany). For amplification of DNA fragments larger than 4 kb, a high-fidelity *Taq* polymerase was used (Platinum *Taq* DNA polymerase high fidelity; Invitrogen). Southern blot analysis was performed as previously described (19). Automated DNA sequencing was performed with the dye deoxy terminator cycle method on an Applied Biosystems 377 sequencer.

Dot blot hybridization. Chromosomal DNA was prepared with the QIAamp DNA mini kit (QIAGEN), and 200-ng samples were spotted onto nylon membranes (Macherey-Nagel). Dot blot hybridization was performed as described previously (19). Probes specific for the meningococcal *hrpA* genes NMB0493, NMB1214, and NMB1768 were generated by PCR using the primer pairs OK99/

OK100 (nucleotides 2050 to 4071), OK103/OK104 (nucleotides 2498 to 4526), and OK105/OK106 (nucleotides 1999 to 4004), respectively. A single probe detecting both NMB0497 and NMB1779 was generated by PCR using the primer pair OK90/OK91 (nucleotides 2482 to 4537). A probe specific for NMC0443 was amplified with the primer pair OK423/OK424 (nucleotides 752 to 1168). A probe specific for NMB1762 was amplified with primer pair OK489/OK490 (nucleotides 2 to 1779). Probes were labeled using a random primer digoxigenin system (Roche, Mannheim, Germany) and detected with the chemiluminescence detection kit (Roche).

RNA extraction and RT-PCR. RNA was prepared using RNeasy kits (QIAGEN), and reverse transcription-PCR was performed with the One Step reverse transcriptase PCR (RT-PCR) kit (QIAGEN). To exclude DNA contamination of the samples, PCR without RT was performed simultaneously. In strain MC58, expression of the *hrpA* genes was analyzed with primer pair OK301/OK302 (NMB0493), OK307/OK308 (NMB1768), OK305/OK306 (NMB1214), and OK303/OK304 (NMB0497/NMB1779), each primer pair amplifying internal fragments of the respective genes. In strain 2120, expression of the *hrpA* gene NMC0444 was analyzed with primer pair OK442/OK443. To analyze the expression of *hrpB* genes homologous to NMB1780 and to simultaneously verify the operon organization of *hrpA* and *hrpB* genes, primer pairs were designed with the forward primer recognizing sequences at the 3' end of *hrpB* and the reverse primer recognizing a conserved region at the 5' end of the associated *hrpA* gene. Strains were analyzed with primer pair OK474/OK475 or OK338/OK339. Expression of *hrpB* genes homologous to NMB1762 was analyzed with primer pair OK491/OK492 (primer sequences are listed in Table S2 in the supplemental material).

Expression in *E. coli* and purification of NMB1779. *E. coli* strain SCS1 containing pQE-NMB1779 (kindly provided by S. Klee) was grown overnight in 10 ml of LB medium containing 100 $\mu\text{g}/\text{ml}$ ampicillin and 12 $\mu\text{g}/\text{ml}$ kanamycin. The following day, 1 ml of the overnight culture was used to inoculate 30 ml of fresh medium. This culture was allowed to grow to an optical density at 600 nm (OD_{600}) of 0.5 to 0.6 before the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to 0.5 mM. After 3 h, the cells were harvested, and the recombinant protein was affinity purified under denaturing conditions by using a Ni-nitrilotriacetic acid spin kit (QIAGEN) according to the manufacturer's instructions.

Rabbit polyclonal antibodies against NMB1779. Rabbit polyclonal antibodies to NMB1779 (RaNMB1779) were raised against the denatured affinity-purified recombinant protein in a New Zealand White female rabbit as previously described (1). RaNMB1779 was used in immunoblots at a dilution of 1:100.

Western blot detection of HrpA proteins. Antibody RaNMB1779 was used to investigate the presence of HrpA proteins. For that purpose, an overnight culture of *N. meningitidis* was inoculated into 20 ml Dulbecco's modified Eagle medium (Biochrom) supplemented with Poly-ViteX (bioMérieux) and grown for 1 h at 37°C and 200 rpm. Cultures were adjusted to an OD_{600} of 0.1 in 20 ml and grown until they reached an OD_{600} of 1.2. For generation of concentrated supernatants, bacteria were pelleted by centrifugation, and the supernatant was passed through a 0.2- μm filter (Whatman). Supernatant proteins were concentrated with an Amicon Ultra-15 centrifugal filter device (Millipore). For generation of whole-cell lysates, cultures were adjusted to an OD_{600} of 0.6 in 1 ml. Bacteria were harvested by centrifugation, and the resulting pellet was resuspended in 50 μl of sample solution. Portions (25 μl) of the respective samples

were used for electrophoresis, blotted, and incubated with the respective antibody. Detection was performed with the SuperSignal West Pico chemiluminescent substrate (Pierce).

Infection experiments. HEp-2 epithelial cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum in a 96-well microtiter plate until confluent growth. FaDu epithelial cells (ATCC HTB-43) were grown in Eagle's minimum essential medium (Cambrex) with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids (Cambrex), 1.0 mM sodium pyruvate (Cambrex), and 10% heat-inactivated fetal calf serum in a 96-well microtiter plate until confluent growth. Prior to infection experiments, cells were washed with phosphate-buffered saline, and infection experiments were performed in RPMI 1640 medium without serum. Infection experiments were performed as described previously (28). The number of cell-associated bacteria was calculated as a percentage of total bacteria present in the well after 5 h of infection. All experiments were performed at least three times unless indicated otherwise. Two-tailed Student's *t* test was used to calculate statistical significance (*P* values).

Protein and nucleic acid sequence analyses. Public databases of published protein and nucleotide sequences were searched using the BLAST programs available at the <http://www.ncbi.nih.gov/BLAST/> and <http://www.tigr.org> websites. The genome databases of meningococcal strain Z2491, MC58, and FAM18 were searched using the BLAST servers available at TIGR (<http://www.tigr.org>) and the Sanger Institute (<http://www.sanger.ac.uk>). Conserved domain searches were performed using SwissPfam available at <http://www.sanger.ac.uk>, InterProScan available at <http://us.expasy.org/prosite/>, and the NCBI Conserved Domain Search available at <http://www.ncbi.nih.gov/>. Protein sequences were aligned using the multiple-sequence alignment ClustalW available at <http://clustalw.ddbj.nig.ac.jp/top-e.html>. Phylogenetic analysis was performed with MEGA (version 3.1) available at www.megasoftware.net.

RESULTS

Presence and distribution of *hrpA* genes among *N. meningitidis* carrier strains. A panel of 822 *N. meningitidis* isolates from healthy individuals was analyzed for the presence of *hrpA* genes. Genetic characteristics of these strains including multilocus sequence typing analysis have been described previously (12). The presence and distribution of *hrpA* genes were analyzed by dot blotting with specific probes for meningococcal *hrpA* genes NMB0493, NMB1214, NMB1768, and NMB0497/1779. A total of 687 strains that gave unambiguous results were analyzed (see Table S3 in the supplemental material). Ninety-seven percent (210/216) of the strains belonging to one of the known hypervirulent lineages were found to be positive for at least one of the probes: 112/114 (98%) isolates belonging to the sequence type 41 and 44 (ST-41/44) complex, all 34 (100%) isolates belonging to the ST-32 complex, 59/63 (94%) isolates belonging to the ST-23 complex, all 3 (100%) isolates belonging to the ST-11 complex, and both (100%) isolates belonging to the ST-8 complex (see Table S3 in the supplemental material). The majority of the remaining strains (334/471 [71%]) not belonging to hypervirulent lineages also carried at least one *hrpA* gene. Among those strains that did not hybridize with the probes, 113/137 (82%) belonged to four of five clonal complexes harboring the capsule null locus (*cnl*) (11) including all strains of the ST-53 complex (52 strains), the ST-198 complex (42 strains), the ST-1117 complex (17 strains), and the ST-1136 complex (2 strains). The majority of *hrpA*-positive strains (255/544 [47%]) hybridized only with the probe specific for NMB0497/NMB1779 (see Table S3 in the supplemental material). Of 544 *hrpA*-positive strains, 159 (29%) strains hybridized with the probes specific for NMB0493, NMB1768, and NMB0497/NMB1779 but not with the probe for NMB1214. More than 50% of these strains were members of the ST-41/44 complex (86 isolates). A positive signal with all four probes

could be demonstrated in 76/544 strains (14%), including most isolates of the ST-32 complex (33/34), all isolates of the ST-269 complex (12 isolates), and a set of strains belonging to the ST-35 complex (10/32 [31%]). Interestingly, the presence of a *hrpA* gene homologous to either NMB0493, NMB1214, or NMB1768 was always associated with a positive signal for NMB0497/NMB1779. Though there were no isolates representing the three serogroup A-related hypervirulent lineages among the carrier strains analyzed, the presence of one *hrpA* gene in the published genome sequence of strain Z2491 suggests that *hrpA* genes are also distributed among the serogroup A strains.

Presence and distribution of *hrpB* genes among *N. meningitidis* carrier strains. Given the high sequence variability of known *hrpA* genes in *N. meningitidis*, strains that did not react with any of the four probes could either completely lack a *hrpA* homologue or contain *hrpA* genes not detected by the probes due to sequence variation. Therefore, additional dot blots were performed with a probe specific for the highly conserved meningococcal *hrpB* gene NMC0443 (Table 1). Almost all strains (801/822 [97.4%]) hybridized with the probe specific for the *hrpB* gene (Fig. 1). As the genes for cognate exoprotein and transporter protein are closely associated in known TPS systems, the presence of genes coding for possible transporter proteins suggests that strains which were negative with the MC58 *hrpA* probes harbored undetected *hrpA* genes with nucleotide sequences different from those present in strain MC58. This hypothesis was tested by PCR of a selection of 30 strains representing hypervirulent clonal complexes (ST-8 complex, ST-11 complex, ST-23 complex, and ST-41/44 complex) and nonhypervirulent clonal complexes (ST-22 complex, ST-60 complex, and ST-162 complex) including those harboring the capsule null locus (ST-53 complex, ST-198 complex, ST-845 complex, and ST-1117 complex). Additionally, the 21 strains which did not hybridize with the *hrpB* probe were tested. The forward primer recognized the 3' terminus of the highly conserved *hrpB* gene (corresponding to nucleotides 1641 to 1663 in NMC0443). The reverse primer recognized a conserved motif within the secretion domain present in all known meningococcal *hrpA* genes (corresponding to nucleotides 501 to 520 in NMC0444). A DNA fragment with the expected size was amplified in all strains with the exception of one isolate belonging to the ST-774 complex (data not shown). NMB1762 has been hypothesized to encode the cognate transporter protein for HrpA_{NMB1768} (46). By dot blot hybridization with a probe specific for NMB1762, 328 out of the 822 strains analyzed (40%) gave a positive result (Fig. 1). Almost all strains harboring NMB1768-homologous genes hybridized also with the probe for NMB1762 (288/294 [97.9%]). In all six negative strains, PCR with primers specific for NMB1762 revealed the presence of this gene. Similarly, the strains that hybridized with the probe for NMB1762 but did not hybridize with the probe for NMB1768 or gave ambiguous results (34/328 [10.3%]) were shown to harbor both genes by PCR with primers specific for NMB1768. The only exception was one isolate of the ST-167 complex that was negative with both the probe and the PCR for NMB1768.

Sequence analysis of *hrpA*_{alpha 14}. To determine whether strains that did not hybridize with the MC58 *hrpA* probes but displayed a positive PCR result with the primer pair for *hrpA*

FIG. 1. DNA dot blot analysis of a selection of carrier strains with probes for the *hrpA* genes NMB0493 (A), NMB1214 (B), NMB1768 (C), and NMB0497/NMB1779 (D) and the *hrpB* genes NMB1762 (E) and NMB0496/NMB1780 (F). Each blot (A to F) shows the results of a selection of 100 carrier isolates (alpha 201 to 300). The order of bacterial strains is the same in all panels. The number below each panel is the percentage of strains out of the 822 strains analyzed that hybridized with the respective probe. The locations of the probes used for hybridization are indicated by black bars above the open rectangles representing the respective *hrpA* and *hrpB* ORFs. The numbers below the open rectangles indicate the sizes (in kilobases) of the ORFs.

and *hrpB* harbor a full-length *hrpA* gene, the complete nucleotide sequence of the single *hrpA* gene of strain alpha 14 (cnl:ST-53) was determined from contigs of a whole-genome sequencing project (C. Schoen et al., unpublished data). The 5,949-bp *hrpA* gene from strain alpha 14 (*hrpA*_{alpha 14}) (NM00398) encodes a protein with a predicted molecular mass of approximately 207 kDa displaying an overall identity to NMB1779 of 50.8%, with the highest degree of similarity at the N terminus (98.8% identity within the first 321 aa) including the TPS domain. Motifs critical for secretion of FHA in *B. pertussis* and conserved in strain MC58 *hrpA* genes are also present in NM00398 (see Fig. S1 in the supplemental material). In contrast, the C terminus was more diverse, displaying only 41.5% sequence identity, which accounts for the failure of the probes derived from strain MC58 to detect *hrpA*_{alpha 14}. *hrpA*_{alpha 14} is most closely related to NMC0444 with an overall nucleotide identity of 51.8% and an overall amino acid identity of 78.6%. A phylogenetic tree was constructed to compare HrpA_{alpha 14} with the other known meningococcal HrpA proteins. Accordingly, meningococcal HrpA proteins can clearly be separated into two groups (Fig. 2). HrpA_{alpha 14} clustered into group I, comprising the single HrpA proteins of meningococcal strains Z2491 and FAM18 together with the two HrpA proteins of strain MC58 encoded by NMB0497 and NMB1779. Group II comprised the three HrpA proteins of strain MC58 encoded by NMB0493, NMB1214, and NMB1768 which have no close homologues in the other two available *N. meningitidis* sequences (Fig. 2). Even HrpA proteins within one of these groups display significant C-terminal sequence variation (Fig. 2). The protein sequences encoded by six putative *hrpA* genes identified in the preliminary whole-genome sequence of *Neisseria lactamica* ST640 (available at www.sanger.ac.uk) were included in the analysis. Only genes encoding HrpA homologous to group II, but not to group I, meningococcal HrpA proteins were detected in *N. lactamica* (Fig. 2).

Expression analysis of *hrpA* and *hrpB* genes in a selection of meningococcal strains. RT-PCR was performed to detect the presence of *hrpA* and *hrpB* transcripts in total RNA isolated from different meningococcal strains. In strain MC58, ex-

pression was confirmed for the *hrpA* genes NMB0493, NMB1214, NMB1768, and NMB0497/NMB1779 and for the *hrpB* genes NMB1780/NMB0496 and NMB1762. As NMB0497 and NMB1779 and their associated putative transporters NMB0496 and NMB1780 display almost 100% sequence identity, differentiation between the genes was not achieved. Using a forward primer recognizing the 3' end of the *hrpB* gene NMB1780 and a reverse primer recognizing a conserved region within the 5' end of the *hrpA* gene

FIG. 2. Phylogenetic tree based on the deduced amino acid sequences of the proteins encoded by the *hrpA* genes of strains MC58, Z2491, FAM18, and alpha 14. In addition, the protein sequences encoded by six putative *hrpA* genes present in the preliminary sequence of *Neisseria lactamica* ST640 were included in the analysis. The meningococcal HrpA proteins clearly cluster into two separate groups. The HrpA protein identified in strain alpha 14 (denoted by NM0_0398 in the figure) is closely related to the group of HrpA proteins encoded by genes homologous to NMB1779. In contrast, the six HrpA proteins identified in *N. lactamica* (denoted by NLA8870, NLA12910, NLA10070, NLA8290, NLA8610, and NLA8550 in the figure) are closely related to the second group of meningococcal HrpA proteins encoded by NMB0493, NMB1214, and NMB1768. BP1879 denotes the ORF encoding the filamentous hemagglutinin of *B. pertussis*. Alignments were performed with ClustalW. Tree construction was performed with MEGA (version 3.1) using the neighbor-joining method. Regions of the alignment containing gaps were excluded from the analysis. The scale bar indicates linkage distance.

FIG. 3. RT-PCR analysis of *hrpA* and *hrpB* expression in strain 2120. (A) Schematic representation of the primer pairs used in panel B, primers OK338/OK339 (OK 338/339), OK500/OK501 (OK 500/501), and OK442/OK443 (OK 442/443). (B) Transcripts of *hrpA* and *hrpB* were detected in total RNA preparations of strain 2120. Additionally, it could be shown that both genes are transcribed into a single mRNA. The following templates were included in the samples: total RNA of strain 2120 (lanes 1 to 3, 5, and 6 to 8), no template (negative control) (lanes 4 and 9), and genomic DNA of strain 2120 (positive control) (lane 10). The primer sets included in the samples were as follows: *hrpB*-specific primers (OK500/OK501 primers [see panel A]) (lanes 1 and 6), *hrpA*-specific primers (OK442/OK443 primers [see panel A]) (lanes 2, 7, and 10), primer pair with the forward primer recognizing the 3' end of the *hrpB* gene and the reverse primer recognizing a conserved region within the 5' end of the adjacent *hrpA* (OK338/OK339 [see panel A]) (lanes 3 and 8), and primers specific for *lgtA* (OK487/OK488, positive control) (lanes 4, 5, 8, and 9). The samples loaded in lanes 6 to 10 were not subjected to the reverse transcription step of the RT-PCR procedure and served as controls to rule out DNA contamination of the RNA templates.

NMB1779, it could be shown that both genes are organized in an operon and transcribed into a single mRNA. Similar results were obtained with strain 2120 harboring only one *hrpA* gene (Fig. 3). Therefore, the typical operon structure of other TPS systems is conserved in *N. meningitidis*. The expression of *hrpA* genes was also analyzed in a subset of the carrier strains representing 11 clonal complexes using the primer pair for *hrpA* and *hrpB*. In all strains, a DNA fragment of the expected size was amplified (data not shown). Similarly, a selection of nine carrier strains representing nine different clonal complexes that had been shown to harbor NMB1762 homologues could be shown to express the respective gene. Therefore, all *hrpA* and *hrpB* genes were found to be transcribed in all meningococcal isolates tested.

Meningococcal HrpA proteins are expressed, proteolytically processed, and secreted in an HrpB-dependent manner. To investigate the expression of meningococcal HrpA proteins, a polyclonal rabbit antiserum was raised against the HrpA protein encoded by NMB1779. For that purpose, His-tagged NMB1779 was expressed and purified in *E. coli*. This antiserum was able to recognize recombinant HrpA protein in a Western blot; the protein migrated to a position of approximately 210 kDa (Fig. 4). However, when Western blotting was performed with whole-cell lysates of strain MC58, no band corresponding in size to the recombinant protein could be detected (Fig. 4). Instead, the antiserum did detect small amounts of a protein of approximately 180 kDa which was absent in Δ NMB0497 Δ NMB1779 mutants. Interestingly, analysis of a Δ *hrpB* mutant (simultaneous deletion of NMB0496 and NMB1780) revealed

FIG. 4. Expression analysis of HrpA proteins by Western immunoblot assays. Proteins were separated on a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with polyclonal antibody R α NMB1779. The arrows indicate the processed (white arrows) and unprocessed (black arrow) HrpA proteins. (A) Western blot of whole-cell lysates of strain MC58, strain 2517, and their respective Δ *hrpA* and Δ *hrpB* mutants. Lanes: 1, purified HrpA; M, molecular mass standard (in kilodaltons); 2, MC58 (wild type); 3, MC58 Δ *asiaD* Δ *hrpA*; 4, MC58 Δ *galE* Δ *hrpA*; 5, MC58 Δ *lgtA* Δ *hrpB*; 6, 2517; 7, 2517 Δ *hrpA*; 8, 2517 Δ *hrpB*. In strain MC58, Δ *hrpA* denotes simultaneous deletion of NMB0497 and NMB1779, whereas Δ *hrpB* denotes simultaneous deletion of NMB0496 and NMB1780. (B) Coomassie blue-stained gel of whole-cell lysates used in panel A. (C) Western blot of culture supernatants of strain 2517 and its respective Δ *hrpA* and Δ *hrpB* deletion mutants as well as the double deletion mutant Δ *hrpA* Δ *hrpB* in comparison to whole-cell lysate of strain 2517 Δ *hrpB*. Lanes: 1, 2517; 2, 2517 Δ *hrpA*; 3, 2517 Δ *hrpB*; 4, 2517 Δ *hrpA* Δ *hrpB*; 5, whole-cell lysate of strain 2517 Δ *hrpB*; 6, 2517. (D) Coomassie blue-stained gel of culture supernatants used in panel C.

a prominent band corresponding in size to the full-length recombinant HrpA protein. Therefore, proteolytic processing of meningococcal HrpA proteins might occur during or after translocation across the outer membrane. To determine whether HrpA proteins are secreted into the environment, whole-cell lysates of strain 2517 and its Δ *hrpA* and Δ *hrpB* deletion mutants and a Δ *hrpA* Δ *hrpB* double deletion mutant were compared with concentrated supernatants of the same strains (Fig. 4). Strain 2517 was chosen for these experiments, as only a single *hrpA* gene and its associated *hrpB* gene are present in this strain. Similar to the results obtained with the Δ *hrpB* mutant of strain MC58, a prominent band of approximately 210 kDa was detected in whole-cell lysates of strain 2517 Δ *hrpB* (Fig. 4). This protein could not be detected in both the Δ *hrpA* mutant and the Δ *hrpA* Δ *hrpB* mutant and most likely represents the unprocessed HrpA protein accumulating in the bacterial cell due to the deletion of its transporter HrpB. As for strain MC58, a band of approximately 180 kDa was detected in whole-cell lysates of strain 2517. In addition, a protein of the same size was detected in the culture supernatants (Fig. 4). This form of HrpA was absent from supernatants and cell lysates of the Δ *hrpA* and Δ *hrpA* Δ *hrpB* mutants. These data provide evidence that HrpA proteins are effectively re-

leased into the culture supernatant in the presence of HrpB. Furthermore, HrpA proteins are processed during or after secretion as indicated by the lower molecular mass of the mature HrpA. A small proportion of the processed HrpA remains associated with the bacterial cell.

Contribution of *hrpA* to the interaction of meningococci with host cells. To investigate whether the presence of different *hrpA* genes in strain MC58 contributes to adherence, mutant NMB0493, NMB1214, and NMB1768 genes were deleted in the unencapsulated strain MC58 Δ *siaD* and the unencapsulated and truncated-LPS strain MC58 Δ *siaD* Δ *lgtA* by insertional inactivation of these genes. As NMB0497 represents an almost identical copy of NMB1779, both genes were inactivated simultaneously in strain MC58 Δ *siaD* and in the unencapsulated and truncated-LPS strain MC58 Δ *siaD* Δ *ABCD* Δ *galE*. Inactivation of *hrpA* genes was confirmed by PCR and Southern blot analysis (data not shown). Unencapsulated and truncated-LPS parental strains were chosen, because capsule expression and the presence of the LPS alpha-chain shields meningococci from interaction with epithelial cells (O. Kurzai et al., unpublished data). Infection experiments were performed to quantify the adherence of the Δ *hrpA* mutants to epithelial cells in comparison to their parental strains. The proportion of cell-associated bacteria (adherent and viable intracellular) was quantified 5 h postinfection (p.i.). No difference could be detected in the proportion of cell-associated bacteria between the *hrpA* mutants and their parental strains (data not shown). These data indicate that adherence to epithelial cells is not mediated by an individual *hrpA* present in MC58. As the possibility that the proteins encoded by the different *hrpA* genes share a common function in the interaction with host cells cannot be excluded, further infection experiments were performed using mutants of serogroup C strain 2120 harboring only a single *hrpA* gene. *hrpA* mutants were constructed in the unencapsulated strain 2120 Δ *siaD* and in the unencapsulated and truncated-LPS strain 2120 Δ *siaD* Δ *lgtA* by insertional inactivation. Irrespective of the parental strain genotype, deletion of *hrpA* or *hrpB* did not influence growth kinetics (data not shown). Whereas no difference in the number of HEp-2 cell-associated bacteria could be detected for strain 2120 Δ *siaD* and its respective *hrpA* mutant, deletion of the *hrpA* gene in the strain with truncated LPS led to a significant decrease in the proportion of cell-associated bacteria 5 h p.i. (16.8% versus 34.4% cell-associated bacteria; $P < 0.01$) (Fig. 5). Similar results were obtained with FaDu epithelial cells (Fig. 5), indicating that HrpA contributes to meningococcal adherence to different epithelial cell lines in an unencapsulated and LPS-truncated background and might be functionally redundant in strains carrying more than one allele. The same results were obtained with three additional independent *hrpA* mutants of strain 2120 Δ *siaD* Δ *lgtA* (data not shown). Additionally, deletion of the *hrpB* gene in strain 2120 Δ *siaD* Δ *lgtA* also led to a significant decrease in the proportion of cell-associated bacteria 5 h p.i. (Fig. 5), thus providing further evidence for the functional relevance of HrpA secretion by its associated HrpB. In contrast to epithelial cells, no difference could be detected in the number of cell-associated bacteria in infection experiments with human dendritic cells (data not shown), suggesting that the adhesive properties of HrpA are cell type specific.

FIG. 5. Adhesion of a Δ *hrpA* deletion mutant and a Δ *hrpB* deletion mutant of strain 2517 Δ *lgtA* to HEp2 (A) and FaDu epithelial cells (B) 5 h p.i. Compared to the wild-type (wt) parental strain, the proportion of cell-associated bacteria was significantly lower in the Δ *hrpA* mutant and in the Δ *hrpB* mutant. Means plus standard deviations (error bars) for 10 independent experiments are indicated. Two-tailed Student's *t* test was used to calculate statistical significance (*P* values).

DISCUSSION

Several large exoproteins in various gram-negative bacteria are predicted to be secreted via the TPS system (23). However, relatively few of these proteins have been investigated in detail. FHA, the most extensively characterized of these exoproteins, is an important virulence factor of *Bordetella* and constitutes the main component of the pertussis vaccine (29). In this study, meningococcal *hrpA* genes encoding high-molecular-weight proteins related to FHA were analyzed. The sequence similarities between FHA and HrpA are restricted to the N terminus comprising the TPS domain which is the hallmark of proteins secreted by the TPS pathway. In contrast, the C-terminal portions of the proteins that have been shown to contain the functional domains in *Bordetella* FHA (31) show no relevant homology between the two species. The conserved TPS domain contains secretion determinants mediating translocation of the respective protein across the outer membrane by its associated transporter termed FhaC in *B. pertussis* (21, 23, 46). The presence of the TPS domain including conserved NPNGI and NPNI motifs in the derived protein sequence of the *hrpA* genes of strains MC58, Z2491, FAM18, and alpha 14 together with the fact that more than 99% of the strains investigated in this study contain genes homologous to *fhaC* suggests the presence of a functional TPS in *N. meningitidis*.

Klee et al. were able to demonstrate the presence of genes homologous to the *hrpA* gene of strain Z2491 in 13 strains mainly representing the known hypervirulent lineages of *N.*

meningitidis (27). In this study, by analyzing a large collection of carrier isolates comprising 51 clonal complexes, we provide evidence that *hrpA* genes are ubiquitously present in *N. meningitidis* and are not restricted to strains of the hypervirulent lineages. We propose to divide the meningococcal *hrpA* genes into two groups according to their degree of sequence similarity. Group I comprises the two almost identical genes NMB0497 and NMB1779 together with the single *hrpA* genes present in strain Z2491 (NMA0688), strain FAM18 (NMC0444), and strain alpha 14 (NM00398). Group II comprises NMB1768, NMB0493, and NMB1214. As no genes closely related to group II had until now been identified in meningococcal strains other than strain MC58, our data for the first time provide evidence that they are not restricted to MC58 or its close relatives. From an evolutionary point of view, it is interesting to notice that only *hrpA* genes homologous to group II were detected in *N. lactamica* strain ST640. These findings are congruent with those of a recent study by Snyder and Saunders (41). The authors were able to demonstrate that genes homologous to NMB0493 and NMB1214 are present in all 13 *N. lactamica* strains investigated using comparative genome hybridization to a pan-*Neisseria* microarray (41). Genes homologous to NMB1768 were detected in a subset of *N. lactamica* strains, whereas genes homologous to NMB1779 could not be detected in *N. lactamica* (41). Similarly, the genome of *N. gonorrhoeae* strain FA 1090 contains an ORF disrupted by multiple stop codons, which otherwise would encode a TpsA protein with homology to NMB1768 (46). Even *hrpA* genes from the same group can show considerable sequence variation in the C terminus, which might be due to a direct recombination-based mechanism for C-terminal exchange between *hrpA* genes and a number of silent cassettes encoding alternative C termini present in the meningococcal genome (4, 46). For example, NMB1779 and *hrpA*_{alpha 14}, both group I meningococcal *hrpA* genes, show a sequence diversity of >49%. It is intriguing to speculate that C-terminal sequence variation might have arisen from selective pressure and could alter the functions of these proteins and modulate the virulence potential of different clonal lineages.

HrpA proteins are expressed and released into the culture supernatant by a HrpB-dependent mechanism. However, in contrast to *B. pertussis* and other known TPS systems, where the exoprotein FHA is rapidly degraded intracellularly in the absence of the transporter FhaC (21) (9, 25), meningococcal HrpA accumulates in the bacteria and remains stable and undegraded if HrpB has been deleted. Our data strongly suggest that the HrpA protein is proteolytically processed during or after secretion. Similar processing has been described for *B. pertussis* (29) and *Haemophilus influenzae* (3). Details of the secretion and processing of HrpA will be addressed in further studies.

Until now, the role of FHA-related proteins in the interaction with the human host has been studied in detail in only a few bacterial species. *B. pertussis* FHA mediates adherence to epithelial cells (20, 37, 45) and macrophages (36, 40) and is required for tracheal colonization in vivo (26). Similarly, the FHA-related proteins of nontypeable *H. influenzae*, HMW1 and HMW2, were shown to mediate binding to macrophages (33) and human epithelial cells (42). Our data clearly show that meningococcal HrpA proteins contribute

to adherence to host cells. A *hrpA* deletion mutant of strain 2120, which harbors only one *hrpA* gene, displayed significantly reduced adherence to different epithelial cells. This effect was observed in an unencapsulated background with short LPS (L8 immunotype). It has previously been shown that capsule expression as well as expression of the LPS alpha-chain can shield meningococci from interaction with host cells (28). Both capsule expression and expression of the LPS alpha-chain are subject to phase variation in meningococci (17, 24), and therefore, capsule-deficient as well as LPS-truncated variants may play a role during colonization and pathogenesis (17, 30). Indeed, the function of Opc, a well-known adhesin and invasin of *N. meningitidis* is also dependent on a lack of capsule expression and LPS truncation (47). Due to the fact that only small amounts of HrpA remain cell associated and can be detected in whole-cell lysates, the possibility that the effect of the *hrpA* or *hrpB* deletion on adhesion is indirect (e.g., by altering outer membrane composition), cannot be excluded. However, no gross changes in expression of outer membrane proteins could be detected in the deletion mutants. The roles of *hrpA* genes with homology to NMB1768, NMB0493, and NMB1214 have not been addressed selectively, as strains harboring one of these genes always possess additional *hrpA* genes homologous to NMB1779/NMB0497. In strain MC58, deletions of a single *hrpA* gene had no effect on adhesion, and although we were able to construct a double deletion of NMB1779 and NMB0497 in MC58, the mutant displayed the same adherence phenotype as the parental strain. This might indicate that *hrpA* genes of both groups are functionally redundant in interaction with epithelial cells despite considerable sequence difference.

In summary, FHA-like proteins (HrpA) as well as FhaC-like proteins (HrpB) are encoded and expressed by virtually all *N. meningitidis* strains belonging to both hypervirulent and non-hypervirulent clonal complexes. HrpA proteins are secreted via transporters encoded by *hrpB* genes and accumulate in the bacteria after deletion of this transporter. However, Western blot analysis as well as the observation that HrpA proteins play a role in adhesion to host cells indicates that a proportion of the secreted HrpA remains surface associated. A similar phenomenon has also been described for another meningococcal adhesin of the autotransporter family, App (16). By analogy to the situation in *B. pertussis*, this renders these proteins interesting candidates for designing potential vaccines against serogroup B meningococci.

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