System Specificity of the TpsB Transporters of Coexpressed Two-Partner Secretion Systems of Neisseria meningitidis

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The two-partner secretion (TPS) systems of Gram-negative bacteria consist of a large secreted exoprotein (TpsA) and a transporter protein (TpsB) located in the outer membrane. TpsA targets TpsB for transport across the membrane via its ~30-kDa TPS domain located at its N terminus, and this domain is also the minimal secretory unit. Neisseria meningitidis genomes encode up to five TpsAs and two TpsBs. Sequence alignments of TPS domains suggested that these are organized into three systems, while there are two TpsBs, which raised questions on their system specificity. We show here that the TpsB2 transporter of Neisseria meningitidis is able to secrete all types of TPS domains encoded in N. meningitidis and the related species Neisseria lactamica but not domains of Haemophilus influenzae and Pseudomonas aeruginosa. In contrast, the TpsB1 transporter seemed to be specific for its cognate N. meningitidis system and did not secrete the TPS domains of other meningococcal systems. However, TpsB1 did secrete the TPS2b domain of N. lactamica, which is related to the meningococcal TPS2 domains. Apparently, the secretion depends on specific sequences within the TPS domain rather than the overall TPS domain structure.

The two-partner secretion (TPS) pathway is a widespread protein secretion route in Gram-negative bacteria which consists of a large and secreted exoprotein (TpsA) of typically more than 100 kDa and a ~60-kDa transporter protein (TpsB) (1). Both proteins are synthesized with an N-terminal signal sequence for transport across the inner membrane via the Sec machinery. After transport to the periplasm, the TpsB transporter integrates in the outer membrane to function in the secretion of TpsA. Targeting of this transporter by the TpsA exoprotein is mediated by a conserved targeting domain, called the TPS domain, which is located downstream of the signal peptide at the N terminus of the mature TpsA (1, 2). TpsA and TpsB molecules are generally encoded in a single operon. The secreted TpsA proteins often function as virulence factors by acting as adhesins or toxins to host cells or other bacteria (2, 3). Neisseria meningitidis (meningococcus) is a Gram-negative diplococcus that survives in the human body by colonizing the nasopharynx. Infection can lead to meningitis and sepsis (4). N. meningitidis genomes encode up to three distinct TPS systems (5). Of these, system 1 is ubiquitous, whereas systems 2 and 3 were significantly more prevalent among isolates of hyperinvasive clonal complexes than those of poorly invasive clonal complexes. Several functions have been attributed to the system 1 TpsAs of N. meningitidis. TpsA1 has been shown to promote adherence to cultured epithelial cell lines derived from the human upper respiratory tract (6). Other in vitro experiments have suggested that TpsA1 contributes to intracellular survival and escape from cultured epithelial cells (7). Recently, based upon sequence comparisons, it was proposed that TPS system 1 of N. meningitidis may act as a contact-dependent bacterial killing system (8), but this has not been confirmed experimentally. TpsAs of the latter class bind to the surface of a cell of a related bacterial species, which is subsequently killed when it does not express an immunity protein. Specific functions have not been attributed to systems 2 and 3.

The N. meningitidis strains that encode all three TPS systems show a similar genetic organization of the TPS open reading frames (ORFs), as exemplified by a comparison of the sequenced genomes of N. meningitidis MC58 and H44/76 (9–12). The strains carry two copies of a genetic island encoding TPS system 1, probably as a result of a duplication event. Both copies contain ORFs encoding TpsBs and TpsAs, but one of the islands encodes a truncated TpsB lacking a signal peptide/sequence which, when expressed, cannot reach the outer membrane. Nevertheless, expression data indicated that both TpsA1 proteins are expressed and secreted (5). The system 2 ORFs are located on two chromosomal islands, and both are linked to one of the two system 1 islands. One island contains a putative operon that includes tpsA2a and tpsB2 (NMB1762), while the other contains a singular tpsA2b. The third system contains only a tpsA, lacks a tpsB, and is located on a separate genetic island. Expression data in vitro are lacking, but antibodies recognizing the TPS domain of the system 3 TpsA have been detected in the serum of a patient recovering from meningococcal disease, which indicates expression during infection (5).

The transport of the secreted TpsA by the TpsB transporter requires the interaction of the TPS domain with TpsB, and, in fact, an isolated TPS domain is the minimal secreted unit of the canonical TpsA filamentous hemagglutinin (FHA) of Bordetella pertussis (FhaB) (13). Recognition of the FhaB TPS domain is very specific, and replacing the cognate TpsB FhaC by a TpsB of another TPS system resulted in blocked secretion (14). Multiple TPS systems coexist in several bacterial species, e.g., Haemophilus influenzae (15), Pseudomonas aeruginosa (16), and Neisseria lactamica and N. meningitidis (35). Our previous results indicated that three TPS systems are coexpressed in N. meningitidis (5). Furthermore, the third neisserial TPS system appears to lack a cognate TpsB, since it has never been identified in analyses of available neisserial genomes (5; unpublished results). Of note, several singular tpsA
genes without a dedicated tpsB gene nearby have been found in other bacterial species, including N. lactamica and P. aeruginosa (5, 16). Therefore, the question of how and if TPS domains are selected by their cognate TpsBs arises. We have investigated the transport of neisserial TPS domains by the available TpsBs, as well as their possible redundancies. We conclude that the TpsB transporter of system 1 was rather selective in its recognition of meningococcal TPS domains. In contrast, the TpsB transporter of system 2 (TpsB2) appears to be less specific in target selection than TpsB1, since it transported TPS domains of H. influenzae 2 (TpsB2) appears to be less specific in target selection than their cognate TpsBs arises. We conclude that the TpsB trans- porter of system 1 was rather selective in its recognition of meningococcal TPS domains. In contrast, the TpsB transporter of sys- tem 2 (TpsB2) appears to be less specific in target selection than TpsB1, since it transported TPS domains of N. meningitidis and N. lactamica, although not those of other species.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** N. lactamica strain 26793, N. meningitidis strain H44/76, its encapsulated derivative HB-1, and the knockout derivatives of HB-1, HB-1 tpsB1::kan, HB-1 tpsB2::kan (5), and HB-1 tpsB1::kan tpsB2::gen, were grown on GC agar (Oxoid) supplemented with Vitox (Oxoid) at 37°C in 5% CO₂. Liquid cultures of N. meningitidis strains were grown at 37°C in tryptic soy broth (Gibco-BRL). Gene disruptions were selected on GC-Vitox plates supplemented with 100 µg/ml ampicillin and/or 10 µg/ml gentamicin. Chloramphenicol was added to a final concentration of 8 µg/ml for plasmid selection. H. influenzae strain A950006 was grown on brain heart infusion (Oxoid) supplemented with 10 µg/ml hemin and 10 µg/ml NAD. The Escherichia coli strains Top10F’ (Invitrogen), DH5α, and MC1061 degP::S210A (17) were grown on Luria-Bertani (LB) broth or agar plates supplemented with 100 µg/ml ampicillin or 30 µg/ml chloramphenicol for plasmid maintenance and with 0.5% glucose for full repression of the lac operator, when appropriate. P. aeruginosa PAO1 was grown on LB agar plates at 37°C.

**Cloning of the TPS constructs.** Truncated tpsA ORFs and combinations of these with tpsB ORFs (Table 1) were cloned into the pEN vector, which is a neisserial expression vector (18). The DNA fragments were obtained by PCR using chromosomal DNA obtained from lysed bacterial cells as the template, the Phusion DNA polymerase (Finnzymes) according to the recommendations of the manufacturer, and the primer combinations that are listed in Table S1 in the supplemental material. To prepare the chromosomal DNA, colonies were scraped from freshly grown plates, resuspended in sterile deionized water to an optical density at 600 nm (OD₆₀₀) of ~2.0, and boiled for 5 min. The lysate was subjected to full-speed centrifugation in a microcentrifuge for 5 min, and 5 µl of the supernatant was added to the PCR mixture. The resulting amplicons were cloned into the pGEM-T cloning vector (Promega) and confirmed the supernatant was added to the PCR mixture. The resulting amplicons were cloned into the pGEM-T cloning vector (Promega) and confirmed for their correct sequence. The DNA fragments were then digested out of the resulting plasmid using the restriction enzymes NdeI and BamHI, resulting in a full-length plasmid of choice that was cut with the same enzymes. This procedure places the tpsB2 ORF downstream of the truncated tpsA in that vector. Because the tpsB1 ORF contained internal EcoRI and Ndel sites, we used a different strategy to obtain combinations of this gene and truncated tpsA genes. First, we introduced by PCR a silent mutation removing the internal NdeI choice that was used in the primers and cloned into the pET11a-truncated tpsB ORF plasmid vector with the same enzymes, yielding the neisserial expression vectors that encoded the truncated tpsA ORFs. All restriction enzymes used for this study were purchased from New England BioLabs. To combine a truncated tpsA with a tpsB, the ORF was obtained by PCR, cloned into pGEM-T, and sequenced. The tpsB2 gene was then digested from the pGEM-T plasmid using the BamHI and EcoRI restriction sites that were included in the primers and cloned into the target pET11a-truncated tpsA plasmid of choice that was cut with the same enzymes. This procedure places the tpsB2 ORF downstream of the truncated tpsA in that vector. Because the tpsB1 ORF contained internal EcoRI and Ndel sites, we used a different strategy to obtain combinations of this gene and truncated tpsA genes. First, we introduced by PCR a silent mutation removing the internal NdeI site in tpsB1 and cloned it into the pGEM-T vector, which was then used as a template for further cloning. The mutated tpsB1 ORF was excised from pGEM-T using BamHI/AlclI and EcoRI/AclI, and these two fragments were ligated into the target pET11a-truncated tpsA1 plasmid digested with BamHI and EcoRI, resulting in a full-length tpsB1 downstream of the truncated tpsA. Subsequently, the lac promoter region from the pEN vector was cloned upstream of the combined ORFs, replacing the Ncol-Apal fragment from the pET11a derivative by that of plasmid pEN100 (encoding the truncated tpsA1 ORF; Table 1). The complete construct was then transferred into the pEN vector using Apal-AatII, yielding the plasmid listed in Table 1.

**Construction of the HB-1 tpsB1::kan tpsB2::gen double-knockout strain.** To construct an HB-1 derivative that lacked the tpsB genes, we performed gene disruption of the tpsB2 gene in the already available HB-1 tpsB1::kan strain. From plasmid pKO-TpsB2::kan (5), which contains a kanamycin resistance cassette between fragments that are located up- and downstream of the tpsB2 gene, we excised the kan cassette using BamHI and

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**TABLE 1 Neisserial expression vectors and other plasmids used in this study**

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<th>Construct</th>
<th>Plasmid name</th>
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<td>Source of gen cassette</td>
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⁴ tr, truncated.
replaced it with a cassette encoding gentamicin resistance from pBSL141 (19).

The construct selected for gene replacement contained the *gen* gene in the same orientation as tpsB2 in the chromosome. *N. meningitidis* HB-1 *tpsB1::kan* was transformed by introducing the tpsB2::gen construct. tpsB2 was subsequently disrupted by homologous recombination, which was verified by PCR using primers annealing up- and downstream of the target gene and immunoblot analysis using specific antibodies against TpsB2.

SDS-PAGE and Western blotting. All SDS-PAGE and Western blotting procedures were carried out as described earlier (5). Briefly, *N. meningitidis* HB-1 cultures were grown for 6 h to an OD$_{600}$ of ~3.0 to 4.0 in the presence of 0.1 to 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) where indicated. Cells were harvested by centrifugation (4,500 × g, 5 min), and the pellet was resuspended in phosphate-buffered saline (PBS; pH 7.4) to a final OD$_{600}$ of 10. Culture supernatants were centrifuged (16,000 × g, 5 min) to remove residual cells, and proteins were precipitated from the supernatants with 5% trichloroacetic acid (TCA) and dissolved in a volume of PBS corresponding to a cell density of an OD$_{600}$ of 100 (concentrated 10 times compared to cells). In some experiments, the supernatants were subjected to centrifugation at 200,000 × g in a benchtop ultracentrifuge (Beckman and Coulter). TCA was added to the supernatant of this step and further treated as described above, whereas the pellet obtained by high-speed centrifugation was dissolved in an equal volume of PBS. Cultures of E. coli strains containing pEN plasmids were grown in LB broth to an OD$_{600}$ of ~0.6. IPTG was added to a final concentration of 1 mM, and incubation was resumed for another 2 h. Samples were collected as described above.

Protein samples were separated on 10% or 4 to 15% SDS-polyacrylamide gels (Bio-Rad) and stained with Coomassie brilliant blue G-250, or proteins were blotted onto nitrocellulose for Western blot analyses. Blots were preincubated in blocking buffer (PBS with 0.5% nonfat dried milk [Protifar; Nutricia, The Netherlands] and 0.1% [vol/vol] Tween 20 [Merck]) for at least 4 h. Sera were diluted 1:5,000 (anti-TPS1 and anti-TPS2a antibodies), 1:10,000 (anti-TpsB1 and anti-TpsB2 antibodies and anti-RmpM monoclonal antibody), or 1:2,000 (anti-His antibody; Biosource International) in blocking buffer and incubated for 1 h. After that, blots were washed three times with blocking buffer and incubated for 1 h with the secondary antibody diluted in blocking buffer. The secondary anti sera were goat anti-rabbit immunoglobulin G serum conjugated to horseradish peroxidase (Biosource International), which was diluted 1:10,000, or anti-mouse immunoglobulin G (Biosource International), which was diluted 1:2,000. The binding of antibodies to the blots was visualized using Lumi-Light Plus substrate (Roche). The relative molecular weight of proteins was deduced from the positions of the bands of the prestained Precision Plus protein standard (Bio-Rad) included in each SDS-polyacrylamide gel.

In silico sequence analyses and structural modeling. Pairwise sequence alignments of TPS domains were performed using amino acid sequences of 283 to 305 residues (see Table S2 in the supplemental material). The length was based upon alignments of the known neisserial tpsA genes to the crystal structure of the FhaB TPS domain (20). The encoded protein sequences of the analyzed *N. meningitidis* tpsA genes were first analyzed for the presence and cleavage site of the signal peptide using the SignalP (version 4.0) program (21), and the ~300-residue stretch downstream of the signal peptide was used for the analyses. The sequences of the *N. lactamica* TPS domains were derived from the sequences of the cloned PCR amplicons. The sequences were compared using the BL2Seq server at http://blast.ncbi.nlm.nih.gov/ using standard parameters. Structure-informed multiple-sequence alignments were performed using the Expresso program at http://tcoffee.crg.cat/apps/tcoffee/index.html (22) and the same sequences used for the pairwise sequence alignments, including the sequences of the three solved TPS domain structures. Models for the structure of the neisserial TPS domains were obtained by submitting the sequences to the Phyre website at http://www.sbg.bio.ic.ac.uk/phyre2/ html/page.cgi?id=index (23). Additional models were obtained from the i-Tasser website at http://zhanglab.ccmb.med.umich.edu/i-TASSER/ (24), but these yielded comparable outcomes. To avoid biases derived from different modeling strategies, we compared only the Phyre-derived models. The models were tested for quality by comparing them to the known structure of the FHA TPS domain using the TM-align program at http://zhanglab.ccmb.med.umich.edu/TM-align/ (25) (see Table S3 in the supplemental material).

Nucleotide sequence accession numbers. GenBank accession numbers for the cloned regions of *N. lactamica* 26793 are KC182755 (TPS-NL2a) and KC182756 (TPS-NL4).

RESULTS

Construction of a minimal TPS expression system in *N. meningitidis*. It had been shown for several TPS systems that a truncated TpsA protein consisting of the signal peptide and the TPS domain is efficiently secreted into the culture supernatant (13, 26–29). Previously, we had assigned TPS domains within the neisserial TpsAs based upon sequence homologies (5). Here, we constructed a set of neisserial expression plasmids encoding C-terminally truncated TpsAs of ~400 amino acid residues encompassing the signal peptide and the predicted TPS domain (Fig. 1). The ORFs

![Figure 1](http://jb.asm.org/journal/figures/fig1.png)
expression and secretion of TPS constructs in *N. meningitidis* HB-1, HB-1 *tpsB1::kan*, and HB-1 *tpsB2::kan*. (A) Immunoblots of cell lysates (C) and concentrated culture supernatants (S) of cells grown in the presence (+) or absence (−) of 1 mM IPTG to induce expression of the TPS construct. The bacterial strains grown are indicated on the top, while the TPS constructs with which the strains were transformed are indicated on the left. The blots were incubated with antisera against the TpsB1, TpsB2, Tps1, and Tps22 domains; the His tag was included in the TPS3 domain as indicated on the right. *α*, the TPS1 domain secreted by the TpsB2 protein. (B) Immunoblot of samples of *N. meningitidis* HB-1 containing the TPS1 construct induced with different amounts of IPTG. The blot was incubated with antiserum against the TPS1 domain. The TPS1 bands are indicated by black arrowheads. Conc. sup., concentrated culture supernatants. (C) Immunoblot of samples of *N. meningitidis* HB-1 containing the TPS1 construct induced or not with IPTG. The blot was incubated with antiserum against the TPS1 domain, focusing on the endogenous full-length TpsA1. The TpsA1 bands in the cell lysates of HB-1 containing the TPS1 construct induced with different amounts of IPTG. The blot was incubated with antiserum against the TPS1 domain. The TPS1 bands are indicated by black arrowheads. The open arrowhead indicates a background band recognized by the anti-TPS1 serum. *α*, the ~240-kDa band accumulating in the cell fractions as a result of overproduction of the TPS1 domain. The positions of the *Mr* markers are indicated on the left in each panel.

Expression of the TPS1 construct resulted in detection of proteins in the cell fractions of HB-1 and its knockout derivatives (Fig. 2). The sizes of the TPS1 and TPS2b domains detected corresponded to the calculated sizes for the constructs after cleavage of the predicted signal peptides (Table 2). The TPS3 domain showed the expected size of 39 kDa, but a 37-kDa band that is perhaps a degradation product was also shown. When we expressed the TPS2a construct, we observed in the supernatant a band of ~39 kDa, which was larger than the expected size of ~34 kDa (Table 2). This might indicate modification of the protein during biogenesis, as has been shown for TPS systems in other bacterial species (30–32) and has also been suggested for neisserial TPS systems (5). Of note, the *tpsA2a* operon includes an ORF encoding a putative glycosyltransferase.

Expression of the TPS1 construct resulted in detection of proteins in the cell fractions of HB-1 and its knockout derivatives (Fig. 2), which in size corresponded to unprocessed (calculated to be ~41 kDa) and processed versions of the TPS1 domain. A similar accumulation had previously been observed for endogenous full-length TpsA1 in the cell fraction of an HB-1 *tpsB1::kan* strain in which its secretion is blocked (5). Cellular intermediates were not detected for the other constructs, suggesting either that these were efficiently secreted or that nonsecreted TPS domains were subjected to intracellular degradation. The recombinant TPS domains were detectable in culture supernatants on Coomassie-stained SDS-polyacrylamide gels (results not shown) for all domains with the exception of TPS3, suggesting a lower level of expression or secretion of this domain. The intracellular accumulation and secretion of the TPS1 domain appeared to be IPTG dose-dependent.

Bands of 32 to 40 kDa were detected in HB-1 culture supernatants (Fig. 2). The sizes of the TPS1 and TPS2b domains detected corresponded to the calculated sizes for the constructs after cleavage of the predicted signal peptides (Table 2). The TPS3 domain showed the expected size of 39 kDa, but a 37-kDa band that is perhaps a degradation product was also shown. When we expressed the TPS2a construct, we observed in the supernatant a band of ~39 kDa, which was larger than the expected size of ~34 kDa (Table 2). This might indicate modification of the protein during biogenesis, as has been shown for TPS systems in other bacterial species (30–32) and has also been suggested for neisserial TPS systems (5). Of note, the *tpsA2a* operon includes an ORF encoding a putative glycosyltransferase.

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Secretion of meningococcal TPS domains. We transformed *N. meningitidis* HB-1, an unencapsulated derivative of H44/76 (18), and its knockout derivatives, HB-1 *tpsB1::kan* and HB-1 *tpsB2::kan* (5), with the constructed plasmids to test the expression and secretion of the TPS domains. Cultures were grown in the presence or absence of 1 mM IPTG, and cells and culture supernatants were collected. Neither the presence of the plasmids nor the induction of gene expression by IPTG influenced the growth curves of the strains. Protein samples were run on SDS-polyacrylamide gels and blotted for immunodetection. Blots incubated with antisera raised against the TpsB1 and TpsB2 proteins confirmed the absence of the transporters in the respective knockout strains (Fig. 2 and results not shown). To analyze the secretion of the respective TPS domains, blots were incubated with antisera against TPS1, TPS2, and the His tag that was attached to TPS3.

were cloned from *N. meningitidis* H44/76 by PCR and placed under the control of an IPTG-inducible promoter (Table 1). The expression vectors included truncated *tpsA1* (TPS1), *tpsA2a* (TPS2a), *tpsA2b* (TPS2b), and *tpsA3* (TPS3) ORFs. Truncated *tpsA1b* was not included, because the encoded signal peptide and TPS domain are identical to those of TpsA1a, except for three residues at the C terminus, which are absent in TpsA1b (5). The TPS3 domain was extended with a C-terminal His tag to enable detection by anti-His antibodies. Sequence analysis of the cloned ORFs and the H44/76 TPS loci in the two available genome sequences (11, 12) confirmed that H44/76 encodes three TPS systems (5). Of note, the *tpsA2a* operon includes an ORF encoding a putative glycosyltransferase.

Expression of the TPS1 construct resulted in detection of proteins in the cell fractions of HB-1 and its knockout derivatives (Fig. 2), which in size corresponded to unprocessed (calculated to be ~41 kDa) and processed versions of the TPS1 domain. A similar accumulation had previously been observed for endogenous full-length TpsA1 in the cell fraction of an HB-1 *tpsB1::kan* strain in which its secretion is blocked (5). Cellular intermediates were not detected for the other constructs, suggesting either that these were efficiently secreted or that nonsecreted TPS domains were subjected to intracellular degradation. The recombinant TPS domains were detectable in culture supernatants on Coomassie-stained SDS-polyacrylamide gels (results not shown) for all domains with the exception of TPS3, suggesting a lower level of expression or secretion of this domain. The intracellular accumulation and secretion of the TPS1 domain appeared to be IPTG dose-dependent.

Secretion of meningococcal TPS domains. We transformed *N. meningitidis* HB-1, an unencapsulated derivative of H44/76 (18), and its knockout derivatives, HB-1 *tpsB1::kan* and HB-1 *tpsB2::kan* (5), with the constructed plasmids to test the expression and secretion of the TPS domains. Cultures were grown in the presence or absence of 1 mM IPTG, and cells and culture supernatants were collected. Neither the presence of the plasmids nor the induction of gene expression by IPTG influenced the growth curves of the strains. Protein samples were run on SDS-polyacrylamide gels and blotted for immunodetection. Blots incubated with antisera raised against the TpsB1 and TpsB2 proteins confirmed the absence of the transporters in the respective knockout strains (Fig. 2 and results not shown). To analyze the secretion of the respective TPS domains, blots were incubated with antisera against TPS1, TPS2, and the His tag that was attached to TPS3.
TABLE 2 Neisserial TPS domains encoded in the neisserial expression vectors

<table>
<thead>
<tr>
<th>Construct</th>
<th>Source</th>
<th>NCBI protein accession no.</th>
<th>TPS length (bp)</th>
<th>Positions</th>
<th>$M_w$</th>
<th>Observed $M_w$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPS1</td>
<td>N. meningitidis H44/76</td>
<td>EFV63548</td>
<td>307</td>
<td>73–379</td>
<td>31.3</td>
<td>32</td>
</tr>
<tr>
<td>TPS2a</td>
<td>N. meningitidis H44/76</td>
<td>EFV64269</td>
<td>325</td>
<td>81–405</td>
<td>33.8</td>
<td>39</td>
</tr>
<tr>
<td>TPS2b</td>
<td>N. meningitidis H44/76</td>
<td>EFV63446</td>
<td>375</td>
<td>78–452</td>
<td>38.9</td>
<td>39</td>
</tr>
<tr>
<td>TPS3</td>
<td>N. meningitidis H44/76</td>
<td>EFV64019</td>
<td>379</td>
<td>78–456</td>
<td>38.9</td>
<td>39/37</td>
</tr>
<tr>
<td>TPS-NL2a</td>
<td>N. lactamica 26793</td>
<td>KC182755</td>
<td>391</td>
<td>76–467</td>
<td>40.7</td>
<td>41</td>
</tr>
<tr>
<td>TPS-NL4</td>
<td>N. lactamica 26793</td>
<td>KC182756</td>
<td>311</td>
<td>78–389</td>
<td>31.1</td>
<td>32</td>
</tr>
<tr>
<td>TPS-HMW1</td>
<td>H. influenzae A950006</td>
<td>AAD56660</td>
<td>298</td>
<td>69–365</td>
<td>32.0d</td>
<td>33</td>
</tr>
<tr>
<td>TPS-PA</td>
<td>P. aeruginosa PA01</td>
<td>NP_249381</td>
<td>323</td>
<td>39–361</td>
<td>34.2d</td>
<td>35</td>
</tr>
</tbody>
</table>

a Expected length of the sequence that folds into the TPS domain after passage of the inner membrane.

b Positions of the TPS sequence in the full-length TpsA.

c Secreted protein compared to protein markers on a 10% SDS-polyacrylamide gel.

d Including a 6× His tag.

This table lists the Neisserial TPS domains encoded in the neisserial expression vectors. The table includes the construct name, the source bacteria, the NCBI protein accession number, the TPS length in base pairs, the positions, the monomeric mass ($M_w$), and the observed mass ($M_w$).

dependent (Fig. 2B). Furthermore, the induction of the TPS1 construct resulted in a competition with secretion of the full-length TpsA1, resulting in the accumulation of an ~240-kDa band in the cell fraction and decrease of detection of the secreted ~240- and ~200-kDa bands in the culture supernatant, similar to the accumulated TpsA1 in the HB-1 tpsB1:kan strain (Fig. 2C). At high levels of IPTG (1 mM), the amount of secreted TPS1 in the culture supernatant seemed to decrease (Fig. 2B), likely also as a result of the competition for TpsB. In contrast, expression of the TPS2a, TPS2b, and TPS3 constructs did not result in a block of the secretion of the full-length TpsA2a and Tps2b proteins (results not shown). Overall, our results indicated that truncated TpsA constructs are expressed and secreted into the neisserial culture medium, similar to what was observed for other TPS systems. Furthermore, our results suggested that the secretion of TpsA3 may not require the expression of a dedicated tpsB.

Specificity for TpsB1 and TpsB2 of meningococcal TPS domains. It has been suggested that recognition of a TPS domain by its TpsB transporter is system specific (1, 14), but this was tested for only a limited number of combinations of TPS domains and TpsB transporters. In fact, the observed secretion of the TPS3 domain suggested a reduced system specificity of one or both of the N. meningitidis TpsBs. We therefore investigated secretion of the TPS domain in strains that lack either TpsB1, TpsB2, or both by transforming HB-1 tpsB1:kan, HB-1 tpsB2:kan (5), and HB-1 tpsB1:kan tpsB2:gen (this study) with the TPS expression vectors and tested the transformants for secretion of the TPS domains.

When the TPS constructs were expressed in the HB-1 tpsB1::kan tpsB2::gen double-knockout strain, none of the TPS domains was detected in the culture supernatant (results not shown and Fig. 3), indicating that a TpsB protein is required for secretion. TPS2a and TPS2b were clearly detected in the culture supernatant of HB-1 tpsB1:kan but not in that of HB-1 tpsB2:kan (Fig. 2), suggesting that the domains are specifically recognized by the TpsB2 transporter and not by TpsB1. Furthermore, the TPS2a and TPS2b domains, which were not secreted in the tpsB2:kan mutant, appeared to be degraded, presumably in the periplasm, since no accumulation of the TPS domains was detected in the cell fractions. Of note, endogenous full-length TpsA2a and TpsA2b had also not been detected in an HB-1 tpsB2:kan background (5). The requirement for TpsB2 for secretion was confirmed when we transformed the HB-1 tpsB1:kan tpsB2::gen strains with a neisserial expression vector that contained both TPS2b (of TpsA2b; Fig. 1) and tpsB2 under the control of the lac promoter (construct TPS2b-TpsB2; Table 1). TpsB2 was detected in the cell fractions of cells harboring this construct, confirming expression (Fig. 3A). We detected TPS2b in the concentrated supernatant fractions of cells that produced TpsB2 from the TPS2b-TpsB2 construct and not in those of HB-1 tpsB1:kan tpsB2::gen cells containing the TPS2b construct. Similar results were obtained with a TPS2a-TpsB2 construct (results not shown). We detected trace amounts of the overproduced TpsB2 protein in the supernatant, suggesting that a low level of leakage of cellular content had occurred. Such leakage may derive from the formation of outer membrane vesicles or blebs, for which N. meningitidis is well-known. To test this supposition and to exclude the possibility that the TPS2 proteins detected in the supernatant did result from mere leakage, we subjected the culture supernatant to ultracentrifugation at 200,000 × g to pellet blebs and cellular debris (Fig. 3A). The results clearly indicated that the TpsB2 protein detected in the culture supernatant was in the high-speed-centrifugation pellet fraction and absent from the high-speed-centrifugation supernatant fraction. Importantly, the TPS2b protein remained almost totally in the high-speed-centrifugation supernatant, indicating secretion rather than leakage. In support, we tested the distribution of the periplasmic protein RmpM over these fractions (see Fig. S1 in the supplemental material). RmpM associates with outer membrane proteins and is thought to connect the outer membrane to the peptidoglycan layer (33). Small amounts of RmpM were detected in the supernatant. Unlike the TPS2b domain, this RmpM protein was found to split between the high-speed-centrifugation pellet and the high-speed-centrifugation supernatant fractions. Overall, the results supported our conclusion that the TPS2b domain is secreted by the TpsB2 protein.

We then analyzed the secretion of the TPS1 construct. The TPS1 domain was detected in the culture supernatant of HB-1 tpsB2:kan knockout strain, as expected (Fig. 2). Surprisingly, we also observed secreted TPS1 in the culture supernatant of HB-1 tpsB1:kan, which suggested that this domain was recognized and secreted by TpsB2, albeit with a lower efficiency. Apparently, the specificity of TpsB2 for recognition of TPS domains is relaxed compared to that of TpsB1. Secretion of TPS1 by TpsB2 was IPTG dose dependent and also observed at the 0.1 mM IPTG concentration (see Fig. S2 in the supplemental material). The secretion by both meningococcal TpsBs was confirmed when we transformed HB-1 tpsB1:kan tpsB2::gen with constructs expressing TPS1 in combination with either tpsB1 or tpsB2 (TPS1-TpsB1 and TPS1-TpsB2, respectively; Fig. 1 and Table 1). HB-1 tpsB1:kan tpsB2::

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cells expressing the TPS1-TpsB1 construct efficiently secreted TPS1 into the culture supernatant (Fig. 3B). However, cells that expressed the TPS1-TpsB2 construct also secreted TPS1 into the culture supernatant. Again, this secretion appeared to be less efficient since the TPS1 protein accumulated in the cell fractions. The secretion of the TPS1 domain by TpsB2 led us to reassess the secretion of full-length TpsA1 in HB-1, HB-1 \( \text{tpsB}^{\text{I::kan}} \), HB-1 \( \text{tpsB}^{\text{I::gen}} \), and HB-1 \( \text{tpsB}^{\text{I::kan tpsB}^{\text{II::gen}}} \). Previously, we had published that in an HB-1 \( \text{tpsB}^{\text{I::kan}} \) knockout strain, full-length TpsA1 accumulated in the cell fraction, while no secreted protein was detected in the culture supernatant. When we reanalyzed secretion of the endogenous full-length TpsA1 and included the analysis of a high-speed-centrifugation supernatant, we observed a pattern similar to that previously published (5). The absence of TpsB1 in both the \( \text{tpsB}^{\text{I::kan}} \) and \( \text{tpsB}^{\text{I::gen}} \) strains resulted in accumulation of a cellular \( \sim 240\text{-kDa} \) form of full-length TpsA1, despite the presence of TpsB2 in the former strain (see Fig. S3A in the supplemental material). The high-speed-centrifugation culture supernatant of these two knockout strains lacked the secreted \( \sim 240\text{-}, \sim 200\text{-}, \) and \( \sim 75\text{-kDa} \) forms of TpsA1. Apparently, the levels of endogenous TpsA1 were too low to be secreted via endogenous TpsB2, or secretion of full-length TpsA1 by TpsB2 is hampered because additional sequences within TpsA1 contribute to the secretion process.

Finally, we tested the secretion of the TPS3 domain in the \( \text{tpsB}^{\text{I::kan}}, \text{tpsB}^{\text{I::gen}}, \) and \( \text{tpsB}^{\text{I::kan tpsB}^{\text{II::gen}}} \) knockout strains. The TPS3 domain was detected in the culture supernatant of the \( \text{tpsB}^{\text{I::kan}} \) strain but not in that of the \( \text{tpsB}^{\text{I::gen}} \) (Fig. 2) or \( \text{tpsB}^{\text{I::kan tpsB}^{\text{II::gen}}} \) strain (Fig. 3C), suggesting efficient secretion by the TpsB2 transporter. We confirmed this observation by transforming HB-1 \( \text{tpsB}^{\text{I::kan tpsB}^{\text{II::gen}}} \) with a neisserial expression vector that coexpressed TPS3 and TpsB2 (TPS3-TpsB2; Table 1). It resulted in the presence of TpsB2 in the cell fraction and the secretion of the TPS3 in the culture supernatant of these strains (Fig. 3C and results not shown). Overall, the results indicated that the TpsB2 transporter of \( N.\ meningitidis \) H44/76 is able to secrete all TPS domains encoded in the meningococcal genome.

**Species specificity of TpsB transporters.** Our results indicated...
a relaxed specificity of the TpsB2 transporter for TPS domains, while TpsB1 appeared to be committed only to the transport of TpsA1 proteins. To investigate the extent of the observed relaxed specificity of TpsB2, we assessed whether TPS domains from systems of other bacterial species could be secreted by N. meningitidis. We first cloned truncated ORFs encoding the signal peptide and the TPS domains of the TpsAs HMW1 of H. influenzae A950006 (15) and PA0690 of P. aeruginosa PAO1 (34) and included a C-terminal His tag for immunodetection (Table 1). In sequence alignments, these TPS domains scored similarity scores of 23 to 33% compared to those for the meningococcal domains (see Table S2 in the supplemental material). After introduction of the constructs into N. meningitidis HB-1, the TPS domains were not detected in samples of the culture supernatants and the whole-cell lysates, suggesting that the ORFs either are not expressed or are degraded in the periplasm (results not shown). We also cloned the neisserial tpsB2 downstream of the truncated ORFs and introduced these constructs in N. meningitidis HB-1 but detected the expression only of the downstream tpsB2 (results not shown). Periplasmic degradation for other secreted proteins had been shown to be mediated via the DegP protease (35–37), which is a major periplasmic protease in E. coli (17). Expression of the constructs TPS-HMW1-TpsB2 and TPS-PA-TpsB2 in E. coli MC1061 degP::S210A, which expresses an inactivated form of DegP, resulted in detection of the TPS domains as well as the TpsB2 proteins, which accumulated mainly in the cellular fractions, although some leakage to the supernatant fractions was observed (see Fig. S4 in the supplemental material). The results indicated that the expression of the constructs was not compromised per se but that secretion in E. coli and N. meningitidis was absent. Clearly, the TpsB2 transporter is not completely promiscuous in transporting TPS domains.

We then investigated the ability of the meningococcal TpsBs to secrete TPS domains of N. lactamica, since this is a closely related bacterial species that occupies the same niche in the human body. Like in N. meningitidis, several TPS systems have been identified in N. lactamica, and not all appear to have a dedicated TpsB encoded (35, 36). Furthermore, sequence comparisons of TPS domains showed that the systems in N. meningitidis and N. lactamica are related (see Table S2 in the supplemental material). To test the secretion of N. lactamica TPS domains in N. meningitidis, we cloned the sequences encoding the signal peptide and TPS domains of tpsA2b (TPS-NL2b) and tpsA4 (TPS-NL4) from N. lactamica strain 26793 into the neisserial expression vector. The TPS proteins were extended by a His tag for detection. The TPS-NL2b construct encodes a TPS domain that is related to TPS2b of N. meningitidis (80% identity, 85% similarity), while that encoded by NL-TPS4 is more distantly related to the N. meningitidis systems (62% identity and 78% similarity to its closest homologue, TPS3). The constructs were introduced into N. meningitidis HB-1, HB-1 tpsB1::kan, HB-1 tpsB2::kan, and HB-1 tpsB1::kan tpsB2::gen. Immunoblot analysis using anti-His antisera on whole-cell lysates and culture supernatants of N. meningitidis cells expressing the constructs showed efficient secretion of TPS-NL2a and TPS-NL4 in HB-1 and HB-1 tpsB1::kan (Fig. 4A), indicating that the meningococcal TpsB2 is efficiently transporting these TPS domains of N. lactamica. Remarkably, we observed secretion of TPS-NL2b but not that of TPS-NL4 in the culture supernatant of HB-1 tpsB2::kan, albeit with a lower efficiency. Apparently, the N. lactamica TPS2 domain (homologous to TPS1 with 44% identity and 61% similarity) contains sequence information that allows recognition by TpsB1, whereas such information is absent in the N. meningitidis TPS2 domains (homologous to TPS1 with 40% identity and 59% similarity for N. meningitidis TPS2a and 40% identity and 57% similarity for N. meningitidis TPS2b). As expected, no secretion was observed in the tpsB1::kan tpsB2::gen double-knockout strain. Similar to the TPS1 domain, the TPS-NL2b domain in the cell fraction appeared to be more stable or less prone to periplasmic degradation (Fig. 4). Therefore, the secretion via TpsB1 could be forced by the higher numbers of TPS-NL2b domains available. However, when we tested whether the secretion of the TPS-NL2b domain would block the secretion of the endogenous full-length TpsA1 in HB-1, we observed that, despite the accumulation of TPS-NL2b in the cell fraction and in contrast to the observations for the TPS1 domain (Fig. 2C), the full-length TpsA1 protein was normally secreted. Accumulation of the TPS-NL2b domain did not result in accumulation of full-length TpsA1 in the cell fraction, and normal levels of the secreted TpsA1 proteins were detected in the supernatant (see Fig. S3B in the supplemental material). Apparently, the recognition and secretion of the TPS-NL2b domain by TpsB1 were specific and efficient enough to allow normal TpsA1 secretion to take place. Overall, the results show that the TpsB2 transporter of N. meningitidis showed relaxed specificity in recognition of TPS domains that is not restricted to N. meningitidis TPS systems. In contrast, TpsB1 showed a more restricted specificity, although it was engaged by the noncognate TPS-NL2b.

**Structural modeling of neisserial TPS domains.** Structurally, the TPS domains are very similar (20, 29, 38). The crystallized domains show a β-helical stem structure to which α-helical and/or β-sheet appendages are attached (Fig. 5). A multiple-sequence alignment incorporating structure-derived information suggested that two major groups of TPS domain structures exist (38): one including the TPS domains of FHA of B. pertussis (20) and hemolysin A of Proteus mirabilis (29) and the other including the TPS domain of HMW1 of H. influenzae (38). Sequence gazing already indicated that the neisserial TPS domains would fall into the FHA/HlyA class of domains. This was corroborated by the results of a multiple-sequence alignment using the Expresso program (22), which incorporates structural information (see Fig. S5 in the supplemental material). This sequence alignment showed a
DISCUSSION

*N. meningitidis* and *N. lactamica* strains encode multiple TPS systems in a complex chromosomal organization that includes the presence of singular *tpsA* genes (5, 10). Here, we investigated the specificity of the meningococcal TpsB proteins for a range of TPS domains. Our results show that the TpsB1 transporter transports its cognate TPS domains and not the other meningococcal TPS domains. In contrast, the TpsB2 transporter is able to secrete all TPS domains encoded in the meningococcal genome, as well as the two *N. lactamica* TPS domains tested. Surprisingly, TpsB1 was also able to secrete an *N. lactamica* TPS domain.

Although many TPS systems are organized in one operon (1, 2), our results clearly indicate that clustering is not necessary for TpsA secretion to occur. Interestingly, it was shown for the *E. coli* O157:H7 OtpA/OtpB TPS system that a TpsB and TpsA need not to be coexpressed for the TpsB to be functional (39). Meningococcal isolates contain up to five different *tpsA* genes distributed over different chromosomal loci, whereas only two *tpsB* genes were found. Based upon the chromosomal location of the *tpsA* and *tpsB* genes (Fig. 1) and the homology of TPS domains (see Table S2 in the supplemental material), we proposed that these genes are part of three systems (35). The *tpsB* genes are both in an apparent operon with a *tpsA* and were designated systems 1 and 2. A singular *tpsA* gene that was located elsewhere on the chromosome was assigned to each of these two systems (Fig. 1). We confirm here that these singular TpsAs can be secreted via their designated TpsBs. The third meningococcal TPS system consists of a singular *tpsA* gene encoding a protein that carries a TPS domain with a sequence distinct from that of the domains in the other systems (see Table S2 in the supplemental material). In the five sequenced *N. meningitidis* genomes that contain a *tpsA3*, no third *tpsB* could be identified (5, 10) (results not shown). Here we show that the TPS3 domain is secreted by TpsB2, which suggests that a cognate TpsB may not be required or may not exist at all. The latter notion is supported by the observation that five sequenced *N. lactamica* genomes contain almost identical *tpsA3* genes, whereas they all lack an obvious *tpsB3* gene (results not shown). Most likely, the system evolved without a dedicated TpsB.

The presence of multiple TPS systems and of singular *tpsA* genes has been described for other bacterial species. For example, strain O35E of *Moraxella catarrhalis* contains a TPS operon encoding the TpsA-like MhaB2 protein and the TpsB-like MhaC protein that colocalize with a gene encoding a TpsA-like protein called MhaB1 in the opposite orientation (40). The perfectly conserved signal peptide and TPS domains within MhaB1 and MhaB2 suggest that they both use the MhaC protein for transport across the outer membrane. A similar organization was found in *Haemophilus ducreyi* for the LspA1 and LspA2 TPS proteins (41). Furthermore, in *Pseudomonas* genomes, multiple TPS systems have been detected, including a singular *tpsA6* gene (16). However, in contrast to the meningococcal singular TpsA3 protein, the TpsA6 protein of *P. aeruginosa* PAO1 is secreted via a dedicated usher that also functions in the secretion of pilins and not via a TpsB protein (42).

The TpsB2 transporter appeared to be promiscuous in the recognition and secretion of different TPS domains, albeit the efficiency appeared to be decreased for the distantly related TPS1 and TPS-NL4 domains. In contrast, the TpsB1 transporter showed a restricted specificity by transporting its cognate TPS1 domain and not the other meningococcal TPS domains. Surprisingly, TpsB1 also secreted the TPS-NL2b domain from *N. lactamica*, whereas on the sequence and predicted structural level, it is very similar to the meningococcal TPS domains that were not secreted by TpsB1 (see Table S2 and Fig. S4 in the supplemental material).

Apparently, the recognition of a TPS domain by TpsB transporters depends on specific motifs or residues within the TPS domains and not on the overall homology between these domains. Within the TpsB proteins, the periplasmic polypeptide transport-associated (POTRA) domains seem to be important for the recog-
nition of the TPS domains, and their deletion renders TpsB inactive (43, 44). The exact interaction interface between the TPS domain and the TpsB protein is unknown and, apparently, cannot be derived from sequence alignments. Structurally, the TPS domains are very similar (20, 29, 38). The crystallized domains show a β-helical stem structure to which α-helical and/or β-sheet appendages are attached (Fig. 5), and the most conserved region within the TPS domains appeared to be critical for the overall structure (20). Structure-informed alignments of the neisserial TPS domains indicated that the differences between them cluster to a specific region in structural models that is outside the β-helical stem (Fig. 5). Exploring the overall similarity of the meningococcal TPS domains, the observed specificity in TpsB selection, and the detected local differences that may confer this specificity could be instrumental in mapping the interaction interface in detail.

For now, we do not know whether the observed redundancy of meningococcal TpsBs for secretion of TPS domains extends to full-length TpsAs. We did not observe secretion of full-length TpsA1 by TpsB2 when expressed at endogenous levels in HB-1. This may be because of expression levels that are too low or because of additional sequences within the TpsA1 that may contribute to the secretion process, as observed for FHA (45). Nevertheless, it has been widely accepted that TPS domains are absolutely required for TpsA secretion, due to their targeting function, and the redundancy of meningococcal TpsBs for TPS domain recognition may not be trivial, because the meningococcal TPS systems are encoded on genomic islands that carry hallmarks of horizontal gene transfer. Furthermore, the presence of specific TPS systems appears to associate with invasive N. meningitidis isolates (5, 46). Our results suggest that TPS systems may be able to use a local TpsB to be secreted and active. For example, we have identified meningococcal disease isolates that lack a tpsB1 but encode a TpsA1 and a TpsB2 (5), which would then allow its secretion. In addition, our analysis of the N. lactamica ST-640 genome shows that it encodes two identical copies of a tpsB gene in an operon with a tpsA. The ORFs were distinct from the known neisserial TPS systems and hence were classified as TPS system 4 (5). The ST-640 strain also encodes full-length TpsA2 and TpsA3 proteins but no tpsB2. Of course, it needs to be confirmed whether the TpsB4 of ST-640 would be able to secrete TpsAs of other systems, but our findings support a model where neisserial TpsAs may use TpsB4 of ST-640 would be able to secrete TpsAs of other systems, but no...


