Structural Determinants of the Interaction between the TpsA and TpsB Proteins in the *Haemophilus influenzae* HMW1 Two-Partner Secretion System

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ABSTRACT

The two-partner secretion (TPS) pathway in Gram-negative bacteria consists of a TpsA exoprotein and a cognate TpsB outer membrane pore-forming translocator protein. Previous work has demonstrated that the TpsA protein contains an N-terminal TPS domain that plays an important role in targeting the TpsB protein and is required for secretion. The nontypeable *Haemophilus influenzae* HMW1 and HMW2 adhesins are homologous proteins that are prototype TpsA proteins and are secreted by the HMW1B and HMW2B TpsB proteins. In the present study, we sought to define the structural determinants of HMW1 interaction with HMW1B during the transport process and while anchored to the bacterial surface. Modeling of HMW1B revealed an N-terminal periplasmic region that contains two polypeptide transport-associated (POTRA) domains and a C-terminal membrane-localized region that forms a pore. Biochemical studies demonstrated that HMW1 engages HMW1B via interaction between the HMW1 TPS domain and the HMW1B periplasmic region, specifically, the predicted POTRA1 and POTRA2 domains. Subsequently, HMW1 is shuttled to the HMW1B pore, facilitated by the N-terminal region, the middle region, and the NPNG motif in the HMW1 TPS domain. Additional analysis revealed that the interaction between HMW1 and HMW1B is highly specific and is dependent upon the POTRA domains and the pore-forming domain of HMW1B. Further studies established that tethering of HMW1 to the surface-exposed region of HMW1B is dependent upon the external loops of HMW1B formed by residues 267 to 283 and residues 324 to 330. These observations may have broad relevance to proteins secreted by the TPS pathway.

IMPORTANCE

Secretion of HMW1 involves a recognition event between the extended form of the HMW1 propiece and the HMW1B POTRA domains. Our results identify specific interactions between the HMW1 propiece and the periplasmic HMW1B POTRA domains. The results also suggest that the process of HMW1 translocation involves at least two discrete steps, including initial interaction between the HMW1 propiece and the HMW1B POTRA domains and then a separate translocation event. We have also discovered that the HMW1B pore itself appears to influence the translocation process. These observations extend our knowledge of the two-partner secretion system and may be broadly relevant to other proteins secreted by the TPS pathway.

Gram-negative bacteria have developed a number of pathways for targeting proteins to an extracellular location (1). Among the most common of these pathways is the two-partner secretion (TPS) system. TPS systems typically consist of a large secreted protein called a TpsA protein (encoded by a *tpsA* gene) and a cognate outer membrane pore-forming translocator protein called a TpsB protein (encoded by a *tpsB* gene) (2). In addition, some TPS systems include a protein that has glycosyltransferase activity and modifies the TpsA protein (3).

TpsA proteins are rich in β structure and are involved in a variety of functions on the bacterial surface or in the extracellular milieu, including adherence, cytotoxic activity, proteolytic activity, iron binding, and contact-dependent growth inhibition (1, 4). All TpsA proteins harbor a conserved $\sim$ 250-amino-acid N-terminal domain required for secretion called a TPS domain (1, 4). Based on existing crystal structures, the TPS domain forms a right-handed β -helix with a few extrahelical motifs (5–8).

TpsB proteins belong to the Omp85/TpsB superfamily involved in insertion of proteins into or translocation of proteins across the outer membranes of Gram-negative bacteria, mitochondria, and chloroplasts (9–11). All members of this family have between one and seven N-terminal polypeptide transport-

associated (POTRA) domains and a C-terminal transmembrane β barrel (12). The *Bordetella pertussis* FhaC protein is the prototypic TpsB protein and has been crystallized, revealing two periplasmic POTRA domains and a 16-stranded β barrel, with a central pore that is occluded by both the N-terminal H1 helix and an extracellular loop designated L6 that folds back in the channel (13). The POTRA domains in FhaC are involved in recognition of the *B. pertussis* filamentous hemagglutinin (FHA) TpsA protein, interacting with the TPS domain and the first part of the repeat-rich central region of FHA (13).

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Nonencapsulated (nontypeable) *Haemophilus influenzae* is a human-specific pathogen that is a common cause of localized respiratory tract disease and initiates infection by colonizing the respiratory epithelium in the nasopharynx (14, 15). Approximately 75% to 80% of isolates express homologous high-molecular-weight proteins called HMW1 and HMW2 that mediate high-level adherence to respiratory epithelial cells and presumably facilitate the process of colonization (16, 17). The HMW1 and HMW2 adhesins are TpsA proteins and are secreted by the HMW1/HMW1B/HMW1C and the HMW2/HMW2B/HMW2C systems, respectively (18). Among these homologous systems, the HMW1 system has been the subject of most mechanistic studies.

HMW1 is synthesized as a preproprotein and is glycosylated in the cytoplasm by the HMW1C glycosyltransferase (19, 20). Subsequently, an N-terminal 68-amino-acid signal sequence directs the preproprotein to the Sec apparatus and is then cleaved (20). The propiece corresponds to amino acids 69 to 441 and directs the proprotein to the HMW1B TpsB protein and is then cleaved (16, 20–22). Following translocation across the outer membrane via HMW1B, the mature adhesin (corresponding to amino acids 442 to 1536) remains noncovalently associated with the bacterial surface, with small amounts released into the culture supernatant (20, 21). Both glycosylation and formation of a disulfide bond between cysteines in the C-terminal 20 amino acids of HMW1 (cysteines 1518 and 1528) are required for HMW1 anchoring to the bacterial surface (19, 23).

In the present study, we sought to define the structural determinants of HMW1 interaction with HMW1B during the transport process and while anchored to the bacterial surface. Modeling of the HMW1B TpsB protein revealed an N-terminal periplasmic region that contains two POTRA domains and a C-terminal membrane-localized region that forms a pore. Biochemical studies demonstrated that HMW1 engages HMW1B via interaction between the HMW1 propiece and the HMW1B periplasmic region, specifically, the predicted POTRA1 domain (residues 28 to 119) and POTRA2 domain (residues 120 to 186). Subsequently, HMW1 is shuttled to the HMW1B pore, facilitated by the N-terminal region, the middle region, and the NPNG motif in the HMW1 propiece. Additional analysis revealed that the interaction between HMW1 and HMW1B is highly specific and is dependent upon both the periplasmic domain and the pore-forming domain of HMW1B. Further studies established that tethering of HMW1 to the surface-exposed region of HMW1B is dependent upon the external loops of HMW1B formed by residues 267 to 283 (loop 3) and residues 324 to 330 (loop 4). The findings in this study may be broadly relevant to proteins secreted by the TPS pathway.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains DH5 α (Invitrogen) and BL21(DE3) (24) are laboratory strains that have been described previously. *E. coli* H10407 is an enterotoxigenic *E. coli* strain that was generously provided by M. Keuhn and has been described previously (25). *E. coli* derivatives were grown on Luria Bertani (LB) agar or in LB broth and were stored at -80°C in LB broth with 20% glycerol. Plasmid selection was performed with kanamycin at a concentration of 25 $\mu\text{g}/\text{ml}$, ampicillin at a concentration of 100 $\mu\text{g}/\text{ml}$, or chloramphenicol at a concentration of 25 $\mu\text{g}/\text{ml}$, as appropriate.

Protein modeling. HMW1B was threaded onto the predicted crystal structure of FhaC (PDB accession no. 2QDZ) using the One-to One

threading program on the Phyre² (protein homology/analogy recognition engine) protein structure prediction server (26).

The homology-based structure alignment of HMW1B and FhaC was based on Protean three-dimensional (3D) DNASTAR rigid-body alignment (27–29). The calculation of root mean square deviation (RMSD) values was based on the Protean 3D DNASTAR alignment algorithm (30). The SABLE protein structure prediction server was used to generate the predicted secondary structure.

Recombinant DNA methods. DNA ligations, restriction endonuclease digestions, and gel electrophoresis were performed according to standard techniques (31). Plasmids were introduced into *E. coli* by electroporation (32).

Plasmid construction. To create pACYC::HMW1_{P-443}, a PCR product that corresponds to DNA beginning 300 bp upstream of the *hmw1A* promoter region, extends to the coding sequence for HMW1 amino acid 443, and contains a 5' XbaI site and a 3' SmaI site was generated. The PCR product was digested with XbaI and SmaI and ligated into XbaI/SmaI-digested pACYC184. To create pASK-IBA2::HMW1-N', a PCR product that corresponds to the coding sequence for HMW1 residues 69 to 441 and contains a 5' EcoRI site and a 3' SacI site was generated. The PCR product was digested with EcoRI and SacI and ligated into EcoRI/SacI-digested pASK-IBA2. To create pASK-IBA12::HMW1-C', a PCR product that corresponds to the coding sequence for HMW1 residues 1369 to 1536 and contains a 5' BamHI site and a 3' SalI site was generated. The PCR product was digested with BamHI and SalI and ligated into BamHI/SalI-digested pASK-IBA12. To create pACYC::HMW1BC, a 4.8-kb fragment containing *hmw1B*, *hmw1C*, and upstream and downstream sequences was excised from pHMW1-15 using NruI and was inserted into NruI-digested pACYC184.

To create pHAT::*hmw1B*, a PCR product that corresponds to the coding sequence for HMW1B residues 28 to 545 and contains a 5' SalI site and a 3' BamHI site was generated. The PCR product was digested with SalI and BamHI and ligated into SalI/BamHI-digested pHAT::Hia₁₋₄₉. To create pHAT::HMW1B₂₈₋₁₈₇, a PCR product that corresponds to the coding sequence for HMW1B residues 28 to 187 and contains a 5' SalI site and a 3' BamHI site was generated. The PCR product was digested with SalI and BamHI and ligated into SalI/BamHI-digested pHAT::Hia₁₋₄₉. To create pHAT::HMW1B₂₈₋₁₈₇::HMW1B₁₉₀₋₅₄₅, a PCR product that corresponds to the coding sequence for HMW1B residues 190 to 545 and contains a 5' BamHI site and a 3' EcoRI site was generated. The PCR product was digested with BamHI and EcoRI and was ligated into BamHI/EcoRI-digested pHAT::HMW1B₂₈₋₁₈₇. The addition of a BamHI site at the junction of the coding sequence for HMW1B₂₈₋₁₈₇ and the coding sequence for HMW1B₁₉₀₋₅₄₅ resulted in a change of residues 188 and 189 in HMW1B from Gly and Lys to Gly and Ser. To create pHAT::HMW1B₂₈₋₁₈₇::EtpB₂₄₅₋₆₀₃, a PCR product that corresponds to the coding sequence for EtpB residues 245 to 603 from *E. coli* strain H10407 and contains a 5' BamHI site and a 3' EcoRI site was generated. The PCR product was digested with BamHI and EcoRI and ligated into BamHI/EcoRI-digested pHAT::HMW1B₂₈₋₁₈₇. To create pHAT::EtpB₄₇₋₂₄₃, a PCR product that corresponds to the coding sequence for EtpB residues 47 to 243 from *E. coli* strain H10407 and contains a 5' SalI site and a 3' BamHI site was generated. The PCR product was digested with SalI and BamHI and ligated into SalI/BamHI-digested pHAT::Hia₁₋₄₉. To create pHAT::EtpB₄₇₋₂₄₃::HMW1B₁₉₀₋₅₄₅, a PCR product that corresponds to the coding sequence for HMW1B residues 190 to 545 and contains a 5' BamHI site and a 3' EcoRI site was digested with BamHI and EcoRI and ligated into BamHI/EcoRI-digested pHAT::EtpB₄₇₋₂₄₃. To create pHAT::EtpB₄₇₋₂₄₃::EtpB₂₄₅₋₆₀₃, the fragment that corresponds to the coding sequence for EtpB residues 245 to 603 and contains BamHI and EcoRI sites was ligated into BamHI/EcoRI-digested pHAT::EtpB₄₇₋₂₄₃. The addition of a BamHI site at the junction of the coding sequence for EtpB₄₇₋₂₄₃ and the coding sequence for EtpB₂₄₅₋₆₀₃ resulted in a change of residue 244 in EtpB from Pro to Gly and Ser.

To create pHMW1B_{POTRA1 + 2}, a PCR product that corresponds to the coding sequence for a methionine followed by HMW1B residues 28 to 186

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Genotype ^a	Source or reference
Strains		
<i>E. coli</i> DH5 α	Φ 80 <i>dlacZ</i> M15 Δ <i>lacU</i> 169 <i>deoR</i> <i>recA</i> <i>endA</i> 1	Invitrogen
<i>E. coli</i> BL21(DE3)	F [−] <i>ompT</i> <i>hdsB</i> _B (<i>r</i> _B [−] <i>m</i> _B [−]) <i>galK</i> (DE3)	24
<i>E. coli</i> H10407	ETEC serotype O78:H11, LT ⁺ LST ⁺	25
Plasmids		
pACYC184	Cloning vector, Cam ^r Tet ^r	42
pHAT10	Cloning vector containing HAT, Amp ^r	Clontech
pT7-7	Cloning vector, Amp ^r	43
pASK-IBA2	Cloning vector, Amp ^r	IBA
pASK-IBA12	Cloning vector, Amp ^r	IBA
pFLAG-CTC	Cloning vector, Amp ^r	Sigma-Aldrich
pET24d	Cloning vector, Kan ^r	Novagen
pHMW1-15	pT7-7 harboring <i>hmw1A</i> , <i>hmw1B</i> , and <i>hmw1C</i> from <i>H. influenzae</i> strain 12	17
pACYC::HMW1 _{P-443}	pACYC184 vector containing <i>hmw1</i> upstream region and HMW1 coding region residues 1–443	This study
pHAT:: <i>hmw1B</i>	pHAT10 vector encoding HMW1B	37
pACYC:: <i>hmw1BC</i>	pACYC184 with <i>Nru</i> I fragment containing <i>hmw1B</i> , <i>hmw1C</i> , and 3' flanking sequence from pHMW1-15	This study
pHAT::Hia _{1–49}	pHAT10 vector containing the <i>hla</i> upstream region and coding sequence for Hia residues 1–49	This study
pASK-IBA2::HMW1-N'	pASK-IBA2 vector encoding HMW1 residues 69–441 (HMW1 propiece)	This study
pASK-IBA12::HMW1-C'	pASK-IBA12 vector encoding HMW1 residues 1369–1536	This study
pHMW1B _{POTRA1} + 2	pFLAG-CTC vector encoding HMW1B residues 27–186 plus 6 $\times$ His tag	This study
pHMW1B _{POTRA1}	pFLAG-CTC vector encoding HMW1B residues 27–119 plus 6 $\times$ His tag	This study
pHMW1B _{POTRA2}	pET24d vector encoding HMW1B residues 120–186 plus FLAG tag	This study
pHMWB-E46AD47A	Derivative of pHAT:: <i>hmw1B</i> encoding HMW1B with glutamic acid residue 46 and aspartic acid residue 47 mutated to alanine	This study
pHMW1-15B/E46AD47A	Derivative of pHMW1-15 encoding HMW1B with glutamic acid residue 46 and aspartic acid residue 47 mutated to alanine	This study
pHMW1B-L91TQ93A	Derivative of pHAT:: <i>hmw1B</i> with leucine residue 91 changed to threonine and glutamine residue 93 changed to alanine	This study
pHMW1-15B/L91TQ93A	Derivative of pHMW1-15 encoding HMWB with leucine residue 91 changed to threonine and glutamine residue 93 changed to alanine	This study
pHMW1B-K108A	Derivative of pHAT:: <i>hmw1B</i> encoding HMW1B with lysine residue 108 mutated to alanine	This study
pHMW1-15/B-K108A	Derivative of pHMW1-15 with HMW1B lysine residue 108 mutated to alanine	This study
pHMW1B-E112A	Derivative of pHAT:: <i>hmw1B</i> encoding HMW1B with glutamic acid residue 112 mutated to alanine	This study
pHMW1-15/B-E112A	Derivative of pHMW1-15 encoding HMW1B with glutamic acid residue 112 mutated to alanine	This study
pHMW1B-Y117A	Derivative of pHAT:: <i>hmw1B</i> encoding HMW1B with tyrosine residue 117 mutated to alanine	This study
pHMW1-15/B-Y117A	Derivative of pHMW1-15 encoding HMW1B with tyrosine residue 117 mutated to alanine	This study
pHMW1B-F148T	Derivative of pHAT:: <i>hmw1B</i> encoding HMW1B with phenylalanine residue 148 mutated to threonine	This study
pHMW1-15/B-F148T	Derivative of pHMW1-15 encoding HMW1B with phenylalanine residue 148 mutated to threonine	This study
pHMW1B-E152A	Derivative of pHAT:: <i>hmw1B</i> encoding HMW1B with glutamic acid residue 152 mutated to alanine	This study
pHMW1-15/B-E152A	Derivative of pHMW1-15 encoding HMW1B with glutamic acid residue 152 mutated to alanine	This study
pHMW1B-M155N	Derivative of pHAT:: <i>hmw1B</i> encoding HMW1B with methionine residue 155 mutated to asparagine	This study
pHMW1-15/B-M155N	Derivative of pHMW1-15 encoding HMW1B with methionine residue 155 mutated to asparagine	This study
pHMW1B-F148TM155N	Derivative of pHAT:: <i>hmw1B</i> encoding HMW1B with double mutation of F148T and M155N	This study
pHMW1-15/B-F148TM155N	Derivative of pHMW1-15 encoding HMW1B with double mutation of F148T and M155N	This study
pACYC::HMW1 _{P-443} Δ 70–86	Derivative of pACYC::HMW1 _{P-443} with a deletion of coding sequence for residues 70–86	This study
pASK-IBA2::HMW1-N' Δ 70–86	Derivative of pASK-IBA2::HMW1-N' with a deletion of coding sequence for HMW1 residues 70–86	This study
pHMW1-15A:: Δ 70–86	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1 residues 70–86	This study
pACYC::HMW1 _{P-443} Δ 150–153	Derivative of pACYC::HMW1 _{P-443} with a deletion of coding sequence for HMW1 residues 150–153 (NPNG)	This study
pASK-IBA2::HMW1-N' Δ 150–153	Derivative of pASK-IBA2::HMW1-N' with a deletion of coding sequence for HMW1 residues 150–153 (NPNG)	This study

(Continued on following page)

TABLE 1 (Continued)

Strain or plasmid	Genotype ^a	Source or reference
pHMW1-15A::Δ150–153	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1 residues 150–153 (NPNG)	This study
pACYC::HMW1 _{P-443} Δ161–199	Derivative of pACYC::HMW1 _{P-443} with a deletion of coding sequence for residues 161–199	This study
pASK-IBA2::HMW1-N'Δ161–199	Derivative of pASK-IBA2::HMW1-N' with a deletion of coding sequence for HMW1 residues 161–199	This study
pHMW1-15A::Δ161–199	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1 residues 161–199	This study
pACYC::HMW1 _{P-443} Δ243–247	Derivative of pACYC::HMW1 _{P-443} with a deletion of coding sequence for residues 243–247	This study
pASK-IBA2::hwm1AΔ243–247	Derivative of pASK-IBA2::HMW1-N' with a deletion of coding sequence for HMW1 residues 243–247	This study
pHMW1-15A::Δ243–247	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1 residues 243–247	This study
pHAT::HMW1B _{28–187} /HMW1B _{190–545}	Derivative of pHAT::Hia _{1–49} with coding sequence for HMW1B residues 28–187 followed by a BamHI restriction site and coding sequence for HMW1B residues 190–545	This study
pHAT::HMW1B _{28–187} /EtpB _{245–603}	Derivative of pHAT::Hia _{1–49} with coding sequence for HMW1B residues 28–187 followed by a BamHI restriction site and coding sequence for EtpB β-barrel residues 245–603 cloned from ETEC strain H10407	This study
pHAT::EtpB _{47–243} /EtpB _{245–603}	Derivative of pHAT::Hia _{1–49} with coding sequence for EtpB residues 47–243 followed by a BamHI restriction site and coding sequence for EtpB β-barrel residues 245–603	This study
pHAT::EtpB _{47–243} /HMW1B _{190–545}	Derivative of pHAT::Hia _{1–49} with coding sequence for EtpB residues 47–243 followed by a BamHI restriction site and coding sequence for HMW1B β-barrel residues 190–545	This study
pHMW1-15B::Δ201–204 (loop 1)	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1B residues 201–204	This study
pHMW1-15B::Δ234–236 (loop 2)	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1B residues 234–236	This study
pHMW1-15B::Δ275–277 (loop 3)	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1B residues 275–277	This study
pHMW1-15B::Δ324–326 (loop 4)	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1B residues 324–326	This study
pHMW1-15B::Δ417–420 (loop 5)	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1B residues 417–420	This study
pHMW1-15B::Δ478–484 (loop 6)	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1B residues 478–484	This study
pHMW1-15B::Δ521–529 (loop 7)	Derivative of pHMW1-15 with a deletion of coding sequence for HMW1B residues 521–529	This study
pHMW1-15B::PSA275–277AAV	Derivative of pHMW1-15 encoding HMW1B with a substitution of residues 275–277	This study
pHMW1-15B::SEL324–326AAA	Derivative of pHMW1-15 encoding HMW1B with a substitution of residues 324–326	This study
pHMW1-15B::275–277 + 324–326	Derivative of pHMW1-15 encoding HMW1B with a substitution of residues 275–277 and 324–326	This study

^a ETEC, enterotoxigenic *E. coli*; LT, heat-labile toxin; LST, heat-stable toxin; Kan, kanamycin; Amp, ampicillin; Cam, chloramphenicol.

and a C-terminal 6× His tag and contains a 5' XhoI site and a 3' BglII site was generated and ligated into pFLAG-CTC (Sigma Chemicals). To create pHMW1B_{POTRA1}, a PCR product that corresponds to the coding sequence for a methionine followed by HMW1B residues 28 to 119 and a C-terminal 6× His tag and contains a 5' XhoI site and a 3' BglII site was generated and ligated into pFLAG-CTC. To create pHMW1B_{POTRA2}, a PCR product that corresponds to the coding sequence for a methionine followed by HMW1B residues 120 to 186 and a C-terminal FLAG tag (DYKDDDK) and contains a 5' BamHI site and a 3' XhoI site was generated and ligated into BamHI/XhoI-digested pET24d.

To create HMW1B POTRA amino acid substitution mutants for secretion assays, site-directed mutagenesis was performed using pHAT::hwm1B. To create HMW1B POTRA amino acid substitution mutants for adherence assays, site-directed mutagenesis was performed using pHMW1-15. To create HMW1B loop deletion and amino acid substitution mutants, site-directed mutagenesis was performed using pHMW1-15. To create HMW1 propiece deletion mutants, site-directed mutagenesis was performed using pACYC::HMW1_{P-443}, pASK-IBA2::HMW1-N', and pHMW1-15. All site-directed mutagenesis was performed using a QuikChange Mutagenesis XL kit (Stratagene).

All recombinant plasmids were assessed by nucleotide sequence analysis to confirm intended mutations and appropriate wild-type sequence.

Expression and purification of the HMW1 propiece and the HMW1B POTRA domains. The HMW1 propiece (residues 69 to 441) was expressed in *E. coli* DH5α/pASK-IBA2::HMW1-N' and recovered from cell sonicates and purified using StrepTrap HP column chromatography (GE Healthcare). The native HMW1 propiece was expressed in *E. coli* DH5α/pASK-IBA2::HMW1-N' + pACYC-hwm1B as a secreted protein and was precipitated from the culture supernatant using ammonium

sulfate and purified using StrepTrap HP column chromatography. The HMW1B POTRA domains were expressed in *E. coli* DH5α/pHMW1B/POTRA1, *E. coli* BL21(DE3)pHMW1B/POTRA2, and *E. coli* DH5α/pHMW1B/POTRA1 plus POTRA2, recovered from cell sonicates, and purified using HisTrap HP column chromatography. The C-terminal region of HMW1 corresponding to residues 1369 to 1536 (HMW1_{1369–1536}) was used as a negative control and was purified from cell sonicates of *E. coli* DH5α/pASK-IBA2::HMW1-C' using StrepTrap HP column chromatography. The HiaBD1 protein was used as another negative control and was purified as described earlier (33).

Cell fractionation and protein analysis. Whole-cell sonicates were prepared by suspending bacterial pellets in 10 mM HEPES (pH 7.4) and sonicating to clarity. Proteins released from the surface of the organism were recovered by precipitating culture supernatants with 10% trichloroacetic acid (TCA) as described previously (34). Total membranes were recovered by centrifugation of cleared bacterial sonicates. Outer membranes were recovered from total membranes on the basis of Sarkosyl insolubility (35). Proteins were resolved by SDS-PAGE using 10% polyacrylamide gels or 4% to 20% gradient gels (Bio-Rad). Western blot analyses were performed with a guinea pig polyclonal antiserum raised against purified HMW1 (antiserum GP104), a guinea pig polyclonal antiserum raised against the purified HMW1 propiece (antiserum GP61), a rabbit polyclonal antiserum raised against recombinant HMW1B (antiserum R948) (21), or a rabbit polyclonal antiserum raised against the histidine affinity tag (HAT) epitope (Clontech).

Far-Western blot and dot-immunoblot assays. Purified HMW1 propiece derivatives were electrotransferred to nitrocellulose membranes or were spotted onto nitrocellulose membranes using a 96-well vacuum manifold. Membranes were then blocked with 0.5% blocking reagent

FIG 1 Modeled structure of HMW1B. Modeling of HMW1B was based on secondary-structure analysis and the crystal structure of the *B. pertussis* FhaC protein. Panel A shows the predicted secondary structure of HMW1B (top) and the established secondary structure of FhaC (bottom). Green regions are β strands, red regions are helices, and navy-blue regions are coils. Amino acids (aa) shaded in yellow represent the predicted POTRA1 domain, amino acids shaded in powder blue represent the predicted POTRA2 domain, amino acids shaded in pink represent the predicted pore, and amino acids shaded in light green represent the helix H1 (FhaC). Panel B shows the modeled structure of HMW1B based on a model rigid-body alignment of HMW1B onto the FhaC crystal structure. The root mean square deviation of modeled HMW1B based on the structure of FhaC is 1.340. The region colored in dark blue represents the POTRA1 domain, the region colored in royal blue represents the POTRA2 domain, the region colored in gray represents the pore, the internal L6 loop is shown in red, and the conserved VRG motif is shown in green. Panel C shows the overlay of the modeled structure of HMW1B and the crystal structure of FhaC. HMW1B is represented in red, and FhaC is represented in blue.

(Roche) in Tris-buffered saline (TBS) (pH 7.4) for 1 h at room temperature. Subsequently, membranes were incubated with 10 μ g/ml of purified HMW1B derivatives diluted in blocking buffer overnight at 4°C and then washed 3 times with TBS–0.05% Tween 20 (TBS–T). Protein–protein interaction was detected using a mouse antiserum against the FLAG tag (Sigma Chemicals) followed by a horseradish peroxidase (HRP)–linked anti-

mouse secondary antiserum (Sigma Chemicals). Membranes were incubated with chemiluminescent HRP substrate and then exposed to film.

Flow cytometry. To assess the quantity of HMW1 associated with the bacterial surface, flow cytometry was performed with guinea pig polyclonal antiserum GP104, as described previously (19). In brief, bacterial strains were grown to an optical density at 600 nm (OD_{600}) of 0.35, recov-

FIG 2 Interaction between the HMW1 propiece and the HMW1B periplasmic domain. The upper panel shows far-Western blot analyses performed by resolving the HMW1 propiece (HMW1_{69–441}) and the HMW1 C terminus (HMW1_{1369–1536}) on an SDS-PAGE gel followed by transfer to a nitrocellulose membrane and overlaying with HMW1B_{POTRA1 + 2}, HMW1B_{POTRA1}, or HMW1B_{POTRA2} and probing with a monoclonal antibody against the FLAG tag. The lower panel shows the Ponceau S-stained nitrocellulose membrane of transferred protein as a loading control.

ered by centrifugation, washed once with phosphate-buffered saline (PBS), resuspended in 4% paraformaldehyde–PBS, and incubated at room temperature for 30 min. Subsequently, bacteria were recovered by centrifugation, washed 2 times with TBS, and resuspended in 0.1% TBS–50 mM EDTA. Antiserum GP104 was added at a dilution of 1:1,000 and incubated at room temperature for 1 h. Bacteria were recovered, washed 2 times with PBS, and then resuspended in PBS in a mixture with 0.1% BSA and anti-guinea pig antibody–Alexa Fluor 488–fluorescein isothiocyanate (FITC) (Life Technologies) at a dilution of 1:200 and incubated for 1 h. Bacteria were washed 2 times with PBS and analyzed by the Duke Flow Cytometry Facility.

SPR. Surface plasmon resonance (SPR) measurements were made using a BIAcore 3000 instrument, and data analyses were performed with BIAevaluation 4.1 software. The ligands (HiaBD1, HMW1B POTRA1 plus POTRA2, HMW1B POTRA1, and HMW1B POTRA2) were immobilized on a CM5 chip using amine coupling reagents according to the manufacturer’s protocol. Roughly 3,000 to 4,000 resonance units (RU) of ligands and 4,000 RU of Hia BD1 as a negative control were immobilized on flow cells. The wild-type and mutant HMW1 propiece analytes of various concentrations (0.5 μg/ml to 40 μg/ml) were made to flow over the immobilized ligands at a 50-μl/min flow rate for 5 min, and the dissociation was followed for 10 min. The ligand surfaces were regenerated

TABLE 2 SPR analysis of HMW1 propiece and HMW1B POTRA domains

HMW1 propiece category ^a	<i>K_d</i> (nM)			
	POTRA1 + POTRA2	POTRA1	POTRA2	POTRA1 + POTRA2 F148T M155N
Cell lysate	122	138	NB	106
Supernatant	912	771	NB	ND

^a Protein isolated from the cell lysate is in an extended form, and protein recovered from the culture supernatant is in the folded form. NB, no binding; ND, not done.

FIG 3 Influence of folding of the HMW1 propiece on interaction with the HMW1B periplasmic domain. Panel A shows the *T_m* from melting curves of the extended form of the HMW1 propiece (purified from cell lysates) and the folded form of the HMW1 propiece (purified from culture supernatants) using the SYPRO orange melting curve assay. Panel B shows dot immunoblots with un-boiled and boiled samples of the extended form of the HMW1 propiece, the folded form of the HMW1 propiece, and the HMW1 C terminus (C-term) (HMW1_{1369–1536}; negative control) overlaid with purified HMW1B_{POTRA1 + 2} and probed with a mouse monoclonal antibody against the FLAG tag.

by injecting 20 μl of glycine (pH 2.0) at a flow rate of 50 μl/minute. The binding response of each analyte to the negative-control surface was subtracted to obtain the specific binding responses of analytes to the ligands. The specific binding sensograms of each analyte at different concentrations were fitted globally to a 1:1 binding model to yield the dissociation constant (*K_d*).

Adherence assays. Adherence assays were performed with Chang epithelial cells (human conjunctiva; ATCC CCL20.2 [Wong-Kilbourne derivative clone 1-5c-4]) as described previously (36). Percent adherence was calculated by dividing the number of adherent CFU by the number of inoculated CFU. Each strain was examined in triplicate in a given assay, and assays were performed a minimum of 3 times.

Secretion assay. Protein secretion by HMW1B or derivatives of HMW1B was assessed as described previously (22).

SYPRO Orange melting curve assay. Protein structures were assessed by measuring melting temperature (*T_m*) based on binding of SYPRO Orange dye (Molecular Probes) and differential scanning fluorimetry. In this assay, as the temperature is increased and the target protein unfolds, SYPRO Orange binds to exposed hydrophobic regions and fluoresces. To determine the *T_m* for the HMW1 propiece, duplicate 1.5-μg samples of purified native and heat-denatured protein were suspended in 50 μl of 100 mM Tris–150 mM NaCl–1 mM EDTA–1 × SYPRO Orange and were seeded into 96-well plates. Plates were heated in a StepOnePlus thermal

FIG 4 Effect of mutations in the HMW1 propiece on interaction with HMW1B. Panel A shows the ribbon crystal structure of the HMW1 propiece and highlights regions that were deleted (residues 70 to 86, 150 to 153 [NPNG], 161 to 169, and 243 to 247). Panel B shows a Western blot of cell lysates (upper blot) and culture supernatants (lower blot) from *E. coli* DH5α expressing HMW1 propiece derivatives plus HMW1B, probing with antiserum GP61 against the HMW1 propiece. The first lane contains samples from *E. coli* DH5α expressing the wild HMW1 propiece without HMW1B. Asterisks indicate the HMW1 propiece, and dots indicate the HMW1B. Panel C shows a dot immunoblot of purified HMW1 propiece derivatives overlaid with purified HMW1B_{POTRA1 + 2} and probed with a mouse monoclonal antibody against the FLAG tag. Panel D shows adherence to Chang epithelial cells by *E. coli* DH5α expressing HMW1 propiece derivatives plus HMW1B and HMW1C. Adherence is displayed as a percentage of the inoculum; data represent the means of the results of three measurements from a representative assay. Asterisks indicate a statistically significant difference from *E. coli* DH5α harboring cloning vector alone ($P < 0.05$ using the unpaired Student *t* test).

cycler at intervals of 1°C from 29°C to 95°C, with a ramping rate of 1°C per min (Applied Biosystems). The filter configurations were set up to accommodate the optimal excitation (Ex) and emission (Em) wavelengths for SYPRO Orange (Ex, 490 nm; Em, 575 nm).

RESULTS

Predicted structure of the HMW1B TpsB protein. In previous work, we used a combination of c-myc epitope tag insertions and cysteine substitution mutagenesis to characterize the structure of HMW1B and found that HMW1B contains an internal periplasmic domain and a C-terminal pore-forming membrane anchor (37). In this study, we used the FhaC crystal structure and secondary-structure analysis to model HMW1B in greater detail (13). As shown in Fig. 1, the periplasmic region of HMW1B is predicted to have 2 POTRA domains designated POTRA1 and POTRA2, defined by residues 28 to 119 and 120 to 186, respectively. The C terminus is predicted to form a 16-stranded β barrel defined by residues 187 to 545. An extracellular loop corresponding to residues 425 to 449 and containing a conserved valine-arginine-glycine (VRG) motif is predicted to fold back into the pore.

Interaction between the HMW1 propiece and HMW1B periplasmic domain. In earlier work, we demonstrated that the HMW1 propiece corresponding to residues 69 to 441 (HMW1₆₉₋₄₄₁, containing the HMW1 TPS domain) is required for secretion of HMW1, presumably reflecting the role of the propiece in targeting of HMW1 to the HMW1B cognate outer membrane protein (20, 21, 37). In addition, we demonstrated that the HMW1B N-terminal region is necessary for secretion of HMW1 (37). In order to address whether the HMW1 propiece interacts directly with the

HMW1B N-terminal region, we began by performing far-Western blot analyses using the purified HMW1 propiece recovered from cell sonicates of *E. coli* DH5α/pASK-IBA2::HMW1-N' and the purified HMW1B periplasmic region recovered from the cytoplasm of *E. coli* DH5α/pHMW1B_{POTRA1 + 2}. As shown in Fig. 2, we observed specific interaction between these proteins. As a negative control, we examined the HMW1 C terminus (HMW1₁₃₆₉₋₁₅₃₆) and the HMW1B periplasmic region and observed no interaction. Further analysis revealed that the HMW1 propiece interacts with a fragment of the HMW1B periplasmic domain that corresponds to POTRA1 and very weakly with a fragment that corresponds to POTRA2 (Fig. 2). Surface plasmon resonance analysis confirmed the results of the far-Western blot analyses and demonstrated high-affinity interaction between the HMW1 propiece and POTRA1 and no significant interaction between the HMW1 propiece and POTRA2 (Table 2).

Structural determinants of HMW1 propiece interaction with HMW1B. To define the structural features of the HMW1 propiece that influence interaction with the HMW1B periplasmic domain, we compared extended and folded forms of the HMW1 propiece. To purify the extended form, we again used *E. coli* DH5α/pASK-IBA2::HMW1-N' and recovered the HMW1 propiece from cell sonicates. To purify the folded form, we used *E. coli* DH5α/pASK-IBA2::HMW1-N' + pACYC-hmw1BC and recovered the HMW1 propiece from the culture supernatant. As shown in Fig. 3A, examination of the melting curves of the extended form and the folded form using the SYPRO orange melting curve assay demonstrated a T_m of 35.2°C for the extended protein and a T_m of 55.1°C

FIG 5 Structural determinants of HMW1B secretion activity. Panel A shows the modeled structure of HMW1B and highlights residues in POTRA1 plus POTRA2 that were mutated. Panel B shows an alignment of the predicted secondary structure of the POTRA domains in HMW1B and the secondary structure of the POTRA domains in FhaC. The residue designations are based on the start codon methionine at position 1. The orange cylinder represents a coil in HMW1B that is absent in FhaC. Residues circled in blue are amino acids in the region between residues 107 and 120. Residues circled in red are amino acids that correspond to residues in FhaC that have an effect on secretion of FHA (amino acids 46 and 47, 91 and 93, and 148 and 152 and 155 in HMW1B correspond to amino acids 95 and 97, 144 and 146, and 199 and 203 and 206 in FhaC, respectively). Panel C shows a Western blot of outer membrane preparations of *E. coli* DH5 α expressing HMW1B derivatives, probing with antiserum R948 against HMW1B. Panel D shows a Western blot of culture supernatants of *E. coli* DH5 α expressing HMW1B derivatives plus HMW1, probing with antiserum GP61 against the HMW1 propiece. Panel E shows adherence to Chang epithelial cells by *E. coli* DH5 α expressing HMW1B derivatives plus HMW1 and HMW1C. Adherence is displayed as a percentage of the inoculum; data represent the means of the results of three measurements from a representative assay. Asterisks indicate a statistically significant difference from *E. coli* DH5 α expressing wild-type HMW1B plus HMW1 and HMW1C ($P < 0.05$ using the unpaired Student t test).

for the folded protein at room temperature, consistent with the conclusion that the extended form is less structured. As a control, we boiled both the extended form and the folded form and found that the T_m for the folded form was markedly decreased, consistent with denaturation and unfolding. Based on dot immunoblot and surface plasmon resonance analysis, the folded form was capable of only limited interaction with HMW1B_{POTRA1 + 2} (Fig. 3B and Table 2), suggesting that the propiece is extended during interaction with HMW1B.

With this information in mind, we generated a series of mutations in the HMW1 propiece based on the alignment of this polypeptide with the TPS domain in FHA and other TpsA proteins. In particular, we generated deletions of a sequence that forms the first 3 β -strands (residues 70 to 86), a sequence that contains the

NPNG motif (residues 150 to 153), a sequence that forms β 13 β 14 β 15 β 16 and α -helix 1 (residues 161 to 199), and a sequence that forms an external loop outside the beta-helical core between β 22 and β 23 (residues 243 to 247), as shown in Fig. 4A. Western analysis of whole-cell sonicates revealed that all of these derivatives were stably expressed and were exported to the periplasm, based on the presence of both the prepropiece and the propiece (after cleavage of the signal peptide) (Fig. 4B). Similarly, a dot immunoblot revealed that all of these derivatives were capable of interacting with the HMW1B periplasmic domain (the POTRA1 plus POTRA2 domains) at wild-type levels (Fig. 4C). In contrast, Western analysis of TCA-precipitated culture supernatants demonstrated that only the construct with deletion of residues 243 to 247 was translocated across the outer membrane (Fig. 4B), sug-

gesting that the N-terminal end, the middle region, and the NPNG motif of the HMW1 propiece are all essential for secretion of HMW1. Consistent with these observations, HMW1 with deletion of residues 243 to 247 was capable of promoting adherence, although at a reduced level relative to wild-type HMW1.

Structural determinants of HMW1B secretion activity. In earlier work, we found that c-myc insertions at amino acid 141, amino acid 149, and amino acid 157 in HMW1B all interfered with secretion of HMW1 and the HMW1 propiece, likely reflecting disruption of the interaction between the HMW1 propiece and HMW1B (37). To extend these results, we began by creating mutations at amino acids 46 and 47 (Glu/Asp to Ala/Ala), 91 and 93 (Leu/Gln to Ala/Ala), 148 (Phe to Thr), 152 (Glu to Ala), and 155 (Met to Asn), amino acids that correspond to residues in FhaC that have an effect on secretion of FHA (38) (Fig. 5A and B). In addition, we created mutations at amino acids 108 (Lys to Ala), 112 (Glu to Ala), and 117 (Tyr to Ala), residues that reside in a region of the HMW1B POTRA1 domain that appears to be critical for secretion of HMW1 (residues 107 to 120; 37) (Fig. 5A and B). As shown in Fig. 5C, based on Western analysis of outer membrane fractions, all of the resulting mutant proteins were localized to the outer membrane similarly to wild-type HMW1B. Combination of the F148T and M155N mutations had no effect on interaction with the HMW1 propiece but eliminated secretion of the HMW1 propiece and abolished HMW1-mediated adherence (Table 2 and Fig. 5D and E).

Specificity of interaction between the HMW1 propiece and HMW1B POTRA domains. Published information suggests that the interaction between TpsA proteins and cognate TpsB proteins is highly specific (39). To define the determinants of this specificity, we created a series of chimeric proteins containing domains of HMW1B and the EtpB outer membrane translocator protein involved in secretion of the enterotoxigenic *E. coli* EtpA exoprotein. EtpA and EtpB are encoded by the *etpABC* gene cluster, which also encodes the EtpC glycosyltransferase in the HMW1C family (25). In particular, we fused the periplasmic region of either HMW1B or EtpB and the pore-forming β -barrel region of either HMW1B or EtpB. As shown in Fig. 6A, all of these chimeras were expressed and were stable in the outer membrane. Further examination revealed that the control chimera HMW1B-HMW1B containing the HMW1B periplasmic domain fused to the HMW1B pore-forming β -barrel domain functioned like wild-type HMW1B, as assessed by secretion of the HMW1 propiece and the ability to support HMW1-mediated adherence (Fig. 6B and C). Similarly, the control EtpB-EtpB chimera functioned like wild-type EtpB and was able to secrete the EtpA TpsA protein (data not shown). The HMW1B-EtpB chimera containing the HMW1B periplasmic domain and the EtpB pore-forming β -barrel domain was able to recognize and secrete the HMW1 propiece, but only at markedly reduced levels (Fig. 6B). Further analysis revealed that the HMW1B-EtpB chimera was able to support minimal HMW1-mediated adherence (Fig. 6C), indicating that the HMW1B and the EtpB pore-forming domains are not interchangeable. An EtpB-HMW1B chimera containing the EtpB periplasmic domain and the HMW1B pore-forming β -barrel domain and an EtpB-EtpB chimera containing the EtpB periplasmic domain and the EtpB pore-forming β -barrel domain were unable to secrete the HMW1 propiece or support HMW1-mediated adherence (Fig. 6B and C), suggesting that HMW1 does not interact with the EtpB periplasmic domain.

FIG 6 Specificity of interaction between HMW1 propiece and HMW1B POTRA domains. Panel A shows a Western blot of outer membrane preparations from *E. coli* DH5 α expressing chimeric proteins containing the periplasmic domain from either HMW1B or EtpB and the pore-forming domain from either HMW1B or EtpB, probing with an antiserum against the HAT epitope. Panel B shows a Western blot of whole-cell lysates (WC) and culture supernatants (S) from *E. coli* DH5 α expressing the HMW1 propiece plus an HMW1B/EtpB chimera protein, probing with antiserum GP61 against the HMW1 propiece. Panel C shows adherence to Chang epithelial cells by *E. coli* DH5 α expressing an HMW1B/EtpB chimera plus HMW1 and HMW1C. Adherence is displayed as a percentage of the inoculum; data represent the means of the results of three measurements from a representative assay. Asterisks indicate a statistically significant difference from *E. coli* DH5 α harboring cloning vector alone ($P < 0.05$ using the unpaired Student *t* test).

Structural determinants of HMW1 tethering to HMW1B. In order to define the structural determinants of tethering of HMW1 to HMW1B on the bacterial surface, we examined derivatives of HMW1B containing short deletions in each of the 7 predicted surface loops (Fig. 7A). All of the deletion constructs were stably expressed and were inserted into the outer membrane (Fig. 7B). Constructs with deletion of residues in either loop 3 (residues 275 to 277) or loop 4 (residues 324 to 326) were associated with decreased HMW1-mediated adherence (Fig. 7C). Mutation of residues 275 to 277 from ProSerAla to AlaAlaVal and residues 324 to 326 from SerGluLeu to AlaAlaAla reproduced the findings associated with deletions in loop 3 and loop 4 (Fig. 7D), consistent with a defect in HMW1 tethering. Further examination of the amino

FIG 7 Structural determinants of HMW1 tethering to HMW1B. Panel A shows the modeled crystal structure of HMW1B highlighting the surface-exposed loops, labeled 1 to 7 and shown in color. Panel B shows a Western blot of outer membrane preparations from *E. coli* DH5 α expressing HMW1B derivatives with deletion of a portion of a surface-exposed loop, probing with antiserum R948 against HMW1B. Panels C and D show adherence to Chang epithelial cells by *E. coli* DH5 α expressing HMW1B derivatives plus HMW1 and HMW1C. Adherence is displayed as a percentage of the inoculum; data represent the means of the results of three measurements from a representative assay. Asterisks indicate a statistically significant difference from *E. coli* DH5 α expressing wild-type HMW1B plus HMW1 and HMW1C ($P < 0.05$ using the unpaired Student t test). Panel E shows flow cytometry results for *E. coli* DH5 α expressing HMW1B derivatives plus HMW1 and HMW1C, focusing on HMW1B surface loop 3 (residues 275 to 277) and loop 4 (residues 324 to 326) and probing with antiserum GP104 against HMW1. % Gated, relative abundance of surface HMW1.

acid substitutions in loop 3 and loop 4 demonstrated diminished surface-associated HMW1 and increased extracellular release of HMW1 and revealed a more dramatic phenotype when loop 3 and loop 4 mutations were combined (Fig. 7E). Deletion of the other

surface-exposed loops had no effect on HMW1 tethering to HMW1B on the bacterial surface or on HMW1-mediated adherence. Together, these results suggest that loop 3 and loop 4 play a key role in HMW1 tethering to the bacterial surface.

DISCUSSION

The HMW1 and HMW2 proteins are the major adhesins in up to 75% to 80% of nontypeable *H. influenzae* strains and are secreted by the TPS pathway. In this study, we examined the interaction between the HMW1 propiece and the HMW1B outer membrane translocator. The HMW1 propiece corresponds to amino acids 69 to 441 and contains the HMW1 TPS domain, an ~250-amino-acid region that is shared among TpsA proteins and is required for secretion.

Our results demonstrated that the HMW1 propiece interacts directly with the HMW1B POTRA1 and POTRA2 domains in the HMW1B periplasmic region, analogous to observations from studies of the *B. pertussis* FHA/FhaC system (13, 38, 39). These results complement our earlier observations that the HMW1B periplasmic region is required for secretion of HMW1 and for HMW1-mediated adherence, presumably reflecting the critical role of the initial interaction and recognition event (37). Similar to findings with FHA and FhaC, efficient interaction between the HMW1 propiece and the HMW1B POTRA domains requires that the propiece is in an extended form rather than a folded form, suggesting that HMW1 remains unfolded or partially unfolded as it transits through the periplasm (40).

In an effort to address the structural elements of the HMW1 propiece that influence interaction with HMW1B, we generated a series of deletion constructs, including deletion of a sequence that forms the N-terminal β -strands (β 1 β 2 β 3) (residues 70 to 86), a sequence that contains the NPNG motif (residues 150 to 153), a sequence that forms internal β -strands (β 13 β 14 β 15 β 16) and α -helix 1 (residues 161 to 199), and a sequence that forms an external loop outside the beta-helical core between β 22 and β 23 (residues 243 to 247). Interestingly, all of these constructs were capable of interacting with the HMW1B periplasmic region, raising questions about the region or regions in the HMW1 propiece that interact with the HMW1B POTRA domains. In contrast, only the construct with deletion of residues 243 to 247 was secreted and was capable of mediating bacterial adherence. Together, these results suggest that the process of translocation of HMW1 involves at least two discrete steps, including initial interaction between the HMW1 propiece and the HMW1B POTRA domains and then a separate translocation event. Furthermore, it appears that the HMW1 propiece participates in both of these steps.

To extend our studies of the HMW1 propiece deletion constructs, we generated mutations in the HMW1B periplasmic region and examined the effect of these mutations on the interaction with the HMW1 propiece and the full-length HMW1 protein. Analysis of the resulting mutants demonstrated that the F148 and M155 mutations in combination in POTRA2 had no effect on interaction with the HMW1 propiece but eliminated secretion of the HMW1 propiece and abolished HMW1-mediated adherence. These findings differ from observations regarding the FhaC periplasmic region, where residues corresponding to F148 and M155 are critical for interaction with the FHA TPS domain (38). Furthermore, these results provide evidence that the HMW1B POTRA domains have a role in recognizing and interacting with the HMW1 propiece and also have a separate role in initiating translocation of HMW1 across the outer membrane.

Previous studies have demonstrated that the interaction between the TpsA protein and the TpsB protein in a given system is highly specific (39). In this study, we examined the determinants of this specificity by creating chimeric proteins containing do-

main of HMW1B and the enterotoxigenic *E. coli* EtpB protein and examining the ability of these chimeras to recognize and secrete HMW1. Our results demonstrated the critical role of the periplasmic domain containing the POTRA domains. In addition, we found that the pore-forming domain also influences this specificity. In particular, the chimeric protein containing the periplasmic domain of HMW1B and the pore-forming domain of EtpB was associated with only low-level secretion of the HMW1 propiece and only low-level HMW1-mediated adherence, emphasizing that not all TpsB pore-forming domains are the same, consistent with recent observations by Baud et al. (41).

In previous work, we found that anchoring of HMW1 to the bacterial surface requires glycosylation and formation of a disulfide bond between cysteines at the extreme C terminus of HMW1 (19, 23). In this study, we examined the structural aspects of HMW1B that influence anchoring of HMW1. Using the modeled structure of HMW1B as a guide, we created small deletions in each of the predicted surface-exposed loops of HMW1. After confirming that these deletions had no deleterious effects on protein stability or insertion into the outer membrane, we found that deletion of residues 275 to 277 in loop 3 and residues 324 to 326 in loop 4 resulted in marked reductions in HMW1 anchoring and HMW1-mediated adherence. Mutation of residues 275 to 277 and 324 to 326 to small nonpolar residues reproduced these results, providing strong evidence that loops 3 and 4 play a critical role in HMW1 anchoring. At this point, the relationship between glycosylation of HMW1 and disulfide bond formation in HMW1 and loops 3 and 4 in HMW1B remains unclear.

In summary, our results suggest that secretion of HMW1 involves a recognition event between the extended form of the HMW1 propiece and the HMW1B POTRA domains. Following this event, the HMW1 propiece influences translocation of HMW1 across HMW1B, perhaps shuttling HMW1 to the HMW1B pore. It is possible that folding of HMW1 promotes dissociation from the HMW1B POTRA domains and initiates the translocation process. The HMW1B POTRA domains also play a role beyond the recognition event between the HMW1 propiece and the HMW1B periplasmic domain, facilitating translocation of HMW1 across the HMW1B pore and perhaps participating in the shuttling of HMW1 to the HMW1B pore. Once HMW1 is delivered to the HMW1B pore, the pore itself appears to influence the translocation process. Ultimately, HMW1 is delivered to the bacterial surface and remains tethered by a combination incorporating the disulfide bond at the C terminus of the protein, glycosylation of the protein, and the surface-exposed loops 3 and 4 in HMW1B.

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REFERENCES

1. Jacob-Dubuisson F, Locht C, Antoine R. 2001. Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. *Mol Microbiol* 40:306–313. <http://dx.doi.org/10.1046/j.1365-2958.2001.02278.x>.
2. Kostakioti M, Newman CL, Thanassi DG, Stathopoulos C. 2005. Mechanisms of protein export across the bacterial outer membrane. *J Bacteriol* 187:4306–4314. <http://dx.doi.org/10.1128/JB.187.13.4306-4314.2005>.
3. McCann J, St Geme JW, III. 2014. The HMW1C-like glycosyltrans-

- ferases—an enzyme family with a sweet tooth for simple sugars. *PLoS Pathog* 10:e1003977. <http://dx.doi.org/10.1371/journal.ppat.1003977>.
4. Jacob-Dubuisson F, Fernandez R, Coutte L. 2004. Protein secretion through autotransporter and two-partner pathways. *Biochim Biophys Acta* 1694:235–257. <http://dx.doi.org/10.1016/j.bbamcr.2004.03.008>.
 5. Clantin B, Hodak H, Willery E, Loch C, Jacob-Dubuisson F, Villeret V. 2004. The crystal structure of filamentous hemagglutinin secretion domain and its implications for the two-partner secretion pathway. *Proc Natl Acad Sci U S A* 101:6194–6199. <http://dx.doi.org/10.1073/pnas.0400291101>.
 6. Yeo H-J, Yokoyama T, Walkiewicz K, Kim Y, Grass S, St Geme JW, III. 2007. The structure of the *Haemophilus influenzae* HMW1 pro-piece reveals a structural domain that is essential for bacterial two-partner secretion. *J Biol Chem* 282:31076–31084.
 7. Weaver TM, Smith JA, Hocking JM, Bailey LJ, Wawrzyn GT, Howard DR, Sikkink LA, Ramirez-Alvarado M, Thompson JR. 2009. Structural and functional studies of truncated hemolysin A from *Proteus mirabilis*. *J Biol Chem* 284:22297–22309. <http://dx.doi.org/10.1074/jbc.M109.014431>.
 8. Baelen S, Desitte F, Clantin B, Villeret V. 2013. Structure of the secretion domain of HxuA from *Haemophilus influenzae*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 69:1322–1327. <http://dx.doi.org/10.1107/S174430911302962X>.
 9. Gentle IE, Burri L, Lithgow T. 2005. Molecular architecture and function of the Omp85 family of proteins. *Mol Microbiol* 58:1216–1225. <http://dx.doi.org/10.1111/j.1365-2958.2005.04906.x>.
 10. Schleiff E, Soll J. 2005. Membrane protein insertion; mixing eukaryotic and prokaryotic concepts. *EMBO Rep* 6:1023–1027. <http://dx.doi.org/10.1038/sj.embor.7400563>.
 11. Soll J, Schleiff E. 2004. Protein import into chloroplasts. *Nat Rev Mol Cell Biol* 5:198–208. <http://dx.doi.org/10.1038/nrm1333>.
 12. Sánchez-Pulido L, Devos D, Genevrois S, Vicente M, Valencia A. 2003. POTRA: a conserved domain in the FtsQ family and a class of beta-barrel outer membrane proteins. *Trends Biochem Sci* 28:523–526. <http://dx.doi.org/10.1016/j.tibs.2003.08.003>.
 13. Clantin B, Delattre AS, Rucktooa P, Saint N, Meli AC, Loch C, Jacob-Dubuisson F, Villeret V. 2007. Structure of the membrane protein FhaC: a member of the Omp85-TpsB transporter superfamily. *Science* 317:957–961. <http://dx.doi.org/10.1126/science.1143860>.
 14. Turk DC. 1984. The pathogenicity of *Haemophilus influenzae*. *J Med Microbiol* 18:1–16. <http://dx.doi.org/10.1099/00222615-18-1-1>.
 15. St Geme JW, III. 1993. Nontypeable *Haemophilus influenzae* disease: epidemiology, pathogenesis, and prospects for prevention. *Infect Agents Dis* 2:1–16.
 16. Barenkamp SJ, Leininger E. 1992. Cloning, expression, and DNA sequence analysis of genes encoding nontypeable *Haemophilus influenzae* high-molecular-weight surface-exposed proteins related to filamentous hemagglutinin of *Bordetella pertussis*. *Infect Immun* 60:1302–1313.
 17. St Geme JW, III, Falkow S, Barenkamp SJ. 1993. High-molecular-weight proteins of nontypeable *Haemophilus influenzae* mediate attachment to human epithelial cells. *Proc Natl Acad Sci U S A* 90:2875–2879. <http://dx.doi.org/10.1073/pnas.90.7.2875>.
 18. St Geme JW, III, Yeo H-J. 2009. A prototype two-partner secretion pathway: the *Haemophilus influenzae* HMW1 and HMW2 adhesin systems. *Trends Microbiol* 17:355–360. <http://dx.doi.org/10.1016/j.tim.2009.06.002>.
 19. Grass S, Buscher AZ, Swords WE, Apicella MA, Barenkamp SJ, Ozchlewski N, St Geme JW, III. 2003. The *Haemophilus influenzae* HMW1 adhesin is glycosylated in a process that requires the HMW1C protein and an enzyme important for lipooligosaccharide biosynthesis. *Mol Microbiol* 48:737–751. <http://dx.doi.org/10.1365-2958.2003.03450.x>.
 20. Grass S, St Geme JW, III. 2000. Maturation and secretion of the nontypeable *Haemophilus influenzae* HMW1 adhesin: roles of the N-terminal and C-terminal domains. *Mol Microbiol* 36:55–67. <http://dx.doi.org/10.1046/j.1365-2958.2000.01812.x>.
 21. St Geme JW, III, Grass S. 1998. Secretion of the *Haemophilus influenzae* HMW1 and HMW2 adhesins involves a periplasmic intermediate and requires the HMW1 and HMW2 proteins. *Mol Microbiol* 27:617–630. <http://dx.doi.org/10.1046/j.1365-2958.1998.00711.x>.
 22. Surana NK, Grass S, Hardy GG, Li H, Thanassi DG, St Geme JW, III. 2004. Evidence for conserved architecture and physical properties of Omp85-like proteins throughout evolution. *Proc Natl Acad Sci U S A* 101:14497–14502. <http://dx.doi.org/10.1073/pnas.0404679101>.
 23. Buscher AZ, Grass S, Heuser J, Roth R, St Geme JW, III. 2006. Surface anchoring of a bacterial adhesin secreted by the two-partner secretion system. *Mol Microbiol* 61:470–483. <http://dx.doi.org/10.1111/j.1365-2958.2006.05236.x>.
 24. Studier FW, Moffatt BA. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* 189:113–130. [http://dx.doi.org/10.1016/0022-2836\(86\)90385-2](http://dx.doi.org/10.1016/0022-2836(86)90385-2).
 25. Fleckenstein JM, Koushik R, Fischer JF, Burkitt M. 2006. Identification of a two-partner secretion locus of enterotoxigenic *Escherichia coli*. *Infect Immun* 74:2245–2258. <http://dx.doi.org/10.1128/IAI.74.4.2245-2258.2006>.
 26. Kelley LA, Sternberg MJE. 2009. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* 4:363–371. <http://dx.doi.org/10.1038/nprot.2009.2>.
 27. Prlc A, Bliven S, Rose PW, Bluhm WF, Bizon C, Godzik A, Bourne PE. 2010. Precalculated protein structure alignments at the RCSB PDB website. *Bioinformatics* 26:2983–2985. <http://dx.doi.org/10.1093/bioinformatics/btq572>.
 28. Ye Y, Godzik A. 2003. Flexible structure alignment by chaining aligned fragment pairs allowing twists. *19(Suppl 2):ii246–ii255*. <http://dx.doi.org/10.1093/bioinformatics/btg1086>.
 29. Zhang Y, Skolnick J. 2005. TM-align: a protein structure alignment algorithm based on the TM-score. *Nucleic Acids Res* 33:2302–2309. <http://dx.doi.org/10.1093/nar/gki524>.
 30. Bagaria A, Jaravine V, Huang YJ, Montelione GT, Guntert P. 2012. Protein structure validation by generalized linear model root-mean-square deviation prediction. *Protein Sci* 21:229–238. <http://dx.doi.org/10.1002/pro.2007>.
 31. Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 32. Dower WJ, Miller JF, Ragsdale CW. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* 16:6127–6145. <http://dx.doi.org/10.1093/nar/16.13.6127>.
 33. Laarmann S, Cutter D, Juehne T, Barenkamp SJ, St Geme JW, III. 2002. The *Haemophilus influenzae* Hia autotransporter harbors two adhesive pockets that reside in the passenger domain and recognize the same host cell receptor. *Mol Microbiol* 46:731–743. <http://dx.doi.org/10.1046/j.1365-2958.2002.03189.x>.
 34. Barenkamp SJ, St Geme JW, III. 1994. Genes encoding high-molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae* are part of gene clusters. *Infect Immun* 62:3320–3328.
 35. Carlone GM, Thomas ML, Rumschlag HS, Sottnek FO. 1986. Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. *J Clin Microbiol* 24:330–332.
 36. Buscher AZ, Burmeister K, Barenkamp SJ, St Geme JW, III. 2004. Evolutionary and functional relationships among the nontypeable *Haemophilus influenzae* HMW family of adhesins. *J Bacteriol* 186:4209–4217. <http://dx.doi.org/10.1128/JB.186.13.4209-4217.2004>.
 37. Surana NK, Buscher AZ, Hardy GG, Grass S, Kehl-Fie T, St Geme JW, III. 2006. Translocator proteins in the two-partner secretion family have multiple domains. *J Biol Chem* 281:18051–18058. <http://dx.doi.org/10.1074/jbc.M600036200>.
 38. Delattre A-S, Saint N, Clantin B, Willery E, Lippens G, Loch C, Villeret V, Jacob-Dubuisson F. 2011. Substrate recognition by the POTRA domains of TpsB transporter FhaC. *Mol Microbiol* 81:99–112. <http://dx.doi.org/10.1111/j.1365-2958.2011.07680.x>.
 39. Jacob-Dubuisson F, Buisine C, Willery E, Renaud-Mongenie G, Loch C. 1997. Lack of functional complementation between *Bordetella pertussis* filamentous hemagglutinin and *Proteus mirabilis* HpmA hemolysin secretion machineries. *J Bacteriol* 179:37731–37735.
 40. Hodak H, Clantin B, Willery E, Villeret V, Loch C, Jacob-Dubuisson F. 2006. Secretion signal of the filamentous hemagglutinin, a model two-partner secretion substrate. *Mol Microbiol* 61:368–382. <http://dx.doi.org/10.1111/j.1365-2958.2006.05242.x>.
 41. Baud C, Guerin J, Petit E, Lesne E, Dupre E, Loch C, Jacob-Dubuisson F. 2014. Translocation path of a substrate protein through its Omp85 transporter. *Nat Commun* 5:5271–5278. <http://dx.doi.org/10.1038/ncomms6271>.
 42. Chang AC, Cohen SN. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* 134:1141–1156.
 43. Tabor S, Richardson CC. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci U S A* 82:1074–1078. <http://dx.doi.org/10.1073/pnas.82.4.1074>.