Structural determinants of the interaction between the *Haemophilus influenzae* Hap autotransporter and fibronectin

Nicole A. Spahich,1† Roma Kenjale,1 Jessica McCann,1 Guoyu Meng,2 Tomoo Ohashi,3 Harold P. Erickson3 and Joseph W. St. Geme, III4

1Department of Pediatrics and Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC, USA
2State Key Laboratory of Medical Genomics, Shanghai Institute of Hematology, Rui-Jin Hospital affiliated to Shanghai JiaoTong University School of Medicine, 197 Ruijin Er Road, Shanghai 200025, PR China
3Department of Cell Biology, Duke University Medical Center, Durham, NC, USA
4Department of Pediatrics, Children's Hospital of Philadelphia and the Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

*Haemophilus influenzae* is a Gram-negative coccobacillus that initiates infection by colonizing the upper respiratory tract. Hap is an *H. influenzae* serine protease autotransporter protein that mediates adherence, invasion and microcolony formation in assays with human epithelial cells and is presumed to facilitate the process of colonization. Additionally, Hap mediates adherence to fibronectin, laminin and collagen IV, extracellular matrix (ECM) proteins that are present in the respiratory tract and are probably important targets for *H. influenzae* colonization. The region of Hap responsible for adherence to ECM proteins has been localized to the C-terminal 511 aa of the Hap passenger domain (HapS). In this study, we characterized the structural determinants of the interaction between HapS and fibronectin. Using defined fibronectin fragments, we established that Hap interacts with the fibronectin repeat fragment called FNIII(1–2). Using site-directed mutagenesis, we found a series of motifs in the C-terminal region of HapS that contribute to the interaction with fibronectin. Most of these motifs are located on the F1 and F3 faces of the HapS structure, suggesting that the F1 and F3 faces may be responsible for the HapS–fibronectin interaction.

INTRODUCTION

Adherence to host tissue is an essential early step in the pathogenesis of most bacterial diseases. Bacteria often encounter epithelial surfaces and utilize surface adhesins to attach to specific epithelial cell receptors. Adherence to epithelial surfaces and entry into epithelial cells may lead to epithelial or basement membrane damage, resulting in exposure of subepithelial tissues (de Bentzmann et al., 1996; Foster & Höök, 1998). Binding to the extracellular matrix (ECM) promotes colonization of deeper, subepithelial tissues and may provide a protected niche for the infecting bacteria (Steukers et al., 2012). The ECM is a complex structure composed of proteins, proteoglycans and glycosaminoglycans, and forms the scaffolding that supports and maintains mammalian cells and tissues. The components of the ECM, such as collagens, laminin and fibronectin, form complex interactions between themselves and also with epithelial cells. These interactions function to form a barrier against penetration by micro-organisms. Many bacteria take advantage of this barrier and express adhesins specific for the different ECM components (Fink et al., 2002; Steukers et al., 2012).

*Haemophilus influenzae* is a Gram-negative bacterium that is often present in the nasopharyngeal flora of children and adults and is a common cause of both localized respiratory infections and systemic disease (Turk, 1984). In previous studies, *H. influenzae* isolates were found to associate preferably with damaged epithelium and the ECM (Read et al., 1991, 1992). *H. influenzae* elaborates a number of surface proteins that promote adherence to epithelial cells and ECM proteins, including the Hap adhesin (Fink et al., 2002, 2003). Hap is a member of a diverse group of virulence proteins called autotransporters and is ubiquitous.
in *H. influenzae* strains (Henderson & Nataro, 2001; Spahich & St Geme, 2011). Autotransporter proteins have three functional domains, including an N-terminal signal peptide, an internal passenger domain and a C-terminal β domain (Henderson et al., 2004). The passenger domain of Hap is called HapS and is responsible for binding to epithelial cells and the ECM proteins fibronectin, laminin and collagen IV (Fink et al., 2002, 2003). The epithelial cell binding and the ECM binding functions of HapS map to two overlapping regions of the passenger domain. The cell-binding region is located in the C-terminal 311 aa of HapS (HapS726–1036), and the ECM-binding region maps to the C-terminal 511 aa of HapS (HapS526–1036) (Fink et al., 2002).

Fibronectin is a large glycoprotein and a component of the ECM, where it forms a complex network with other ECM proteins, provides attachment sites for cell surface receptors, and helps in cell migration and tissue maintenance (Mosher & McKeown-Longo, 1985; Potts & Campbell, 1994). Fibronectin is a dimer composed of two identical chains that are connected by disulphide bonds at the C-terminus. Structurally, fibronectin is composed of three types of repeating domains or modules called FNII, FNIII and FNIII (Petersen et al., 1983). The modules form functional domains that bind different ligands, including ECM molecules such as collagens, heparin, fibrin and integrins (Pankov & Yamada, 2002). A variety of pathogenic bacteria are capable of binding to fibronectin, with examples including *Mycobacterium* spp., *Escherichia coli*, *Staphylococcus* spp., *Streptococcus* spp., and *Salmonella enterica* Typhimurium (Jonsson et al., 1991; Kingsley et al., 2002; Lindgren et al., 1992; Signäs et al., 1989).

In this study, we used defined fragments of fibronectin to precisely map the domains that are recognized by HapS. Our findings indicate that HapS binds to the fibronectin FNIII1–2 repeat fragment. To identify residues in the Hap ECM binding domain involved in this interaction, we compared the Hap sequence to other well-known fibronectin binding proteins (FnBPs) and found several common motifs. When we mutated these motifs in the Hap passenger domain, bacterial binding to fibronectin was impaired.

### Table 1. Bacterial strains and plasmids

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<tr>
<th>Strain or plasmid</th>
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<tr>
<td><em>E. coli</em> DH5α</td>
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<tr>
<td><em>H. influenzae</em> DB117</td>
<td>Laboratory strain, rec-1, capsule-deficient serotype d, with nonsense mutation in <em>hap</em></td>
<td>Setlow et al. (1968)</td>
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<td><strong>Plasmids</strong></td>
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<td>pLS88</td>
<td><em>E. coli–</em> <em>H. influenzae</em> shuttle vector, Km′, Sm′, Su′</td>
<td>Willson et al. (1989)</td>
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<tr>
<td>pGJB103</td>
<td><em>E. coli–</em> <em>H. influenzae</em> shuttle vector, Tc′</td>
<td>Tomb et al. (1989)</td>
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<td>pHapS243A</td>
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<td><strong>pHap</strong></td>
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<td>pHapA26-525</td>
<td>pLS88 with <em>hap</em> having deletion in residues 26–525</td>
<td>Fink et al. (2003)</td>
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<td>Fink et al. (2003)</td>
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<td>Fink et al. (2003)</td>
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<tr>
<td>pHapETD570AAA</td>
<td>pHapA26-525 with residues 570–572 changed to alanine</td>
<td>This study</td>
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<tr>
<td>pHapTED588AAA</td>
<td>pHapA26-525 with residues 588–590 changed to alanine</td>
<td>This study</td>
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<td>pHapEIV640AAA</td>
<td>pHapA26-525 with residues 640–642 changed to alanine</td>
<td>This study</td>
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<td>pHapEN655AAA</td>
<td>pHapA26-525 with residues 655–656 changed to alanine</td>
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<td>pHapA26-525 with residues 570–572 and 588–590 changed to alanine</td>
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<td>pHapGG597TT</td>
<td>pHapA26-525 with residues 597–598 changed to threonine</td>
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<td>pHapA26-525 with residues 661–662 changed to threonine</td>
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<td>pHapGG661TT+EN655AAA</td>
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<td>This study</td>
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was significantly impaired. However, none of the mutations eliminated binding, suggesting that there may be multiple points of interaction between Hap and fibronectin. Further examination of the Hap$_S$ crystal structure suggests that the F1 and F3 faces of the prism-like structure may be the sites of Hap$_S$–fibronectin interactions.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *H. influenzae* strains were stored at $-80\, ^\circ\text{C}$ in brain heart infusion broth with 20% (v/v) glycerol and were grown either on brain heart infusion agar supplemented with haem and NAD (BHIs) with the appropriate antibiotics or in BHIs broth as described previously (Anderson et al., 1972). *E. coli* DH5$\alpha$ strains were stored at $-80\, ^\circ\text{C}$ in Luria–Bertani (LB) broth with 20% (v/v) glycerol and were grown on LB agar or in LB broth. Kanamycin was used at a concentration of 25 mg ml$^{-1}$ for *H. influenzae* and 50 mg ml$^{-1}$ for *E. coli*.

**Purification of Hap$_S$ protein.** The Hap passenger domain with the S243A mutation (Fink & St Geme, 2003) was purified from culture supernatants of *H. influenzae* strain DB117/pLS88:Hap$_D_{1036-99}$/pGJBI03:HapS243A. Overnight cultures of strain DB117/pLS88:Hap$_D_{1036-99}$/pGJBI03:HapS243A were used to inoculate 6 l BHIs. Cultures were shaken at 37 $^\circ\text{C}$ and grown to an OD$_{600}$ of 0.8. Bacteria were pelleted, and the supernatant was concentrated to $\sim$500 ml using a transverse flow cartridge with a 50 kDa cut-off (Millipore) into 20 mM sodium phosphate (pH 6.4) buffer. The concentrated supernatant was applied to a Sepharose cation-exchange column (GE healthcare) equilibrated with 20 mM sodium phosphate (pH 6.4) buffer. Protein was eluted with a linear sodium chloride gradient (pH 6.4, 0–1 M NaCl), and peak fractions containing Hap$_S$ were pooled and confirmed by Western blot analysis. The protein sample was mixed with ammonium sulfate with a final concentration of 1.3 M and was then loaded onto a phenyl Sepharose column (GE healthcare) equilibrated with buffer containing 20 mM MES (pH 6.4), 1.3 M ammonium sulfate. Fractions were eluted with a reverse ammonium sulfate gradient (pH 6.4, 1–0 M ammonium sulfate). Fractions containing Hap$_S$S243A were pooled and dialysed into a buffer containing 20 mM Tris (pH 7.0), 50 mM NaCl. The peak fraction was estimated to be $\sim$95% pure, as indicated by Coomassie blue-stained SDS-PAGE gels.

**Construction of mutant Hap derivatives.** The plasmid pHapA26-525 (Fink et al., 2003), a pLS88 (Willson et al., 1989) derivative containing the hap gene with a deletion of the coding sequence for residues 26–525 of the Haps protein, was used as a template for PCR. Site-directed mutagenesis was performed using the Quick Change XL II kit from Stratagene according to the manufacturer’s instructions.

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**Fig. 1.** Hap$_S$ binds to FNIII$_{1-2}$. (a) Schematic representation of the domain structure and interactions of fibronectin. (b) Far-Western dot blot assay showing binding of purified Hap$_S$ to immobilized purified full-length fibronectin or fibronectin fragments. FN full and FN 45 are commercial full-length and 45 kDa gelatin-binding domain fragments from Sigma. (c) Far-Western dot blot assay showing binding of purified Hap$_S$ to immobilized purified full-length fibronectin or fibronectin fragments.
Mutations were confirmed by sequencing. After mutagenesis, the resulting plasmid was introduced into *H. influenzae* strain DB117 using the MII/MIV transformation method (Steinhart & Herriott, 1968). To confirm that the deletions did not affect the stability of Hap, outer-membrane proteins from the resulting DB117 strains were examined by Western blot analysis for equivalent quantities of Hap relative to DB117 expressing wild-type Hap.

**Western blot analysis.** Bacteria were grown to an OD$_{600}$ of 0.8, and Sarkosyl-insoluble outer-membrane proteins were isolated as described by Carlone *et al.* (1986). Outer membrane fractions were resuspended in 15 μl 10 mM HEPES (pH 7.4) plus 5 μl 4 × Laemmli buffer. Protein samples were resolved by SDS-PAGE on 10% polyacrylamide gels (Laemmli, 1970), and similar volumes from cultures of similar density were loaded into each well. Resolved proteins were electrotransferred to a nitrocellulose membrane and examined by immunoblot analysis with antisera diluted 1 : 1000. Rab289 antiserum was raised against a C-terminal Hap fragment corresponding to residues 996–1395 and reacts with both Hap$_S$ and Hap$_B$ (the C-terminal membrane-anchoring domain of Hap). IgG rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) was used at a dilution of 1 : 5000. Detection of antibody binding was accomplished by incubating membranes in a chemiluminescent substrate solution (Pierce) and then exposing the membranes to film.

**Far-Western dot-blot assay.** Commercial fibronectin fragments were obtained from Sigma (45 kDa F0162, full-length F2006). Fibronectin fragments were spotted onto nitrocellulose membranes using a 96-well vacuum manifold and incubated for 30 min at room temperature. The membranes were then blocked with 5% (w/v) milk in Tris-buffered saline (TBS; pH 7.4) for 1 h, incubated by shaking with 10 μg ml$^{-1}$ purified Hap$_S$ diluted in blocking buffer for 1 h at 4 °C, and then washed five times with TBS. Hap$_S$ binding was detected using antiserum Rab289 raised against purified Hap$_S$ (1 : 1000 dilution) followed by a horseradish peroxidase (HRP)-linked anti-rabbit secondary antiserum (Sigma). Membranes were incubated with chemiluminescent HRP substrate and then exposed to film.

**Generation of anti-DB117 antibody.** The rabbit antiserum Rab288 was raised against an acetone dried powder preparation of *H. influenzae* strain DB117 and is reactive with strain DB117 whole bacteria.

**ELISA for binding to ECM proteins.** Ninety-six-well ELISA plates (Costar) were coated overnight at 4 °C with fibronectin fragments at 1 μg per well diluted in PBS, pH 7.4. In the case of full-length fibronectin, pre-coated plates were obtained from BD Biosciences. The plates were blocked with 2% (w/v) BSA in PBS for 2 h. Derivatives of *H. influenzae* strain DB117 expressing either full-length
(a) MIVNHNTQAANVITGNES
   IVLPNGNNINKLDYRKEIAY
   NGWFGETKKNHNGRLNL1Y
   KPTEDRTLLSSGGTNKGD
   ITQTGKLFFSGRPHTHAYN
   HLNKRWSEMEPIPOGIEIVWD
   HDWINRTKALFQIKGQSA
   VVSRNVSSIEGNWTVSNNAN
   ATFGVVPNQQNTICTRSDWT
   GLTTCQKVDLTDTKVINSIP

(b) Absorbance at 405 nm

(c) Absorbance at 405 nm

(d) Absorbance at 405 nm
Hap or mutant constructs were grown in 3 ml BHIs to an OD_{600} of 0.4–0.5. One millilitre of the bacterial suspension was centrifuged and then resuspended in 1 ml fresh BHIs. Fifty microlitres of this suspension was added to each well in triplicate and incubated on a plate shaker at 37 °C for 1 h. The wells were washed five times with PBS, and bacterial binding was detected using a 1:1000 dilution of Rab288 in PBS plus 0.1 % BSA. Incubation with the primary antisera was performed at room temperature for 1.5 h. After washing five times with PBS, the wells were incubated with a 1:5000 dilution of anti-rabbit HRP conjugate in PBS plus 0.1 % BSA for 1 h at room temperature. The wells were washed five times with PBS before adding the HRP substrate and reading the absorbance at 405 nm.

RESULTS

HapS binds to fibronectin fragment FNIII(1–2)

Fibronectin can be cleaved by enzymes such as thermolysin into defined fragments that retain adhesive function. The functional domains of fibronectin have been mapped using this technique (Fig. 1a) (Pankov & Yamada, 2002). Previously, we found that HapS binds to the 45 kDa gelatin-binding domain but not the 30 kDa heparin-binding domain of fibronectin (Fink et al., 2002). To extend this result and precisely map the region of fibronectin recognized by Hap, we used Far-Western analysis to test the binding of purified HapS (containing a serine-to-alanine mutation at residue 243 in the protease active site) to a more defined set of purified fibronectin fragments encompassing the FNI, FNII and FNIII domains: FNI(1–9), FNII(1–2), FNIII(1–5), FNIII(4–7), FNIII(7–10), FNIII(10–12), FNIII(12–14) and FNIII(13–15), which had been created previously (Ohashi & Erickson, 2005, 2011). As shown in Fig. 1(b), HapS bound to FNIII(1–5), a fragment that has not been shown previously to react with bacteria. To further map the binding target in fibronectin, we tested fragments FNIII(2–5), FNIII(3–5), FNIII(3–3), FNIII(1–2), FNIII(1) and FNIII(2) and found that HapS bound to FNIII(1–3) and FNIII(1–2) but not to FNIII(2–5), FNIII(2–3) or FNIII(3–5), suggesting that the fibronectin binding region lies in the FNIII(1–2) repeats (Fig. 1c). HapS did not bind to FNIII(1) or FNIII(2) alone by this method, suggesting that FNIII repeats 1 and 2 together are necessary for this interaction.

Bacteria expressing HapΔ26–525 bind fragment FNIII(1–2)

The C-terminal 511 aa of HapS (residues 526–1036) have been determined previously to be necessary and sufficient for full binding to fibronectin as well as laminin and collagen IV (Fink et al., 2003). With this information in mind, we examined H. influenzae DB117/HapA26–525 and DB117/HapΔ26–725 (lacking a portion of the region required for full ECM binding) in assays with full-length fibronectin and FNIII(1–2) immobilized on 96-well plates. As shown in Fig. 2, DB117/HapA26–525 adhered at high levels to FNIII(1–2), similar to results with full-length fibronectin, while DB117/HapΔ26–725 adhered at background levels.

Mutational analysis of putative fibronectin-binding residues in HapS

Because binding to fibronectin requires Hap residues between amino acids 525 and 725, we focused on this region to identify motifs involved in fibronectin binding. Many Gram-positive bacterial fibronectin binding proteins require groups of acidic residues in their fibronectin binding domains (Schwarz-Linek et al., 2003; Westerlund & Korhonen, 1993). We searched for similar motifs in Hap between residues 525 and 725 and identified 570–572/ETD, 588–590/TED, 640–642/EIV and 653–656/EN, all of which are surface exposed in the Hap crystal structure (Fig. 3a). To assess the role of these residues, we mutated them to alanines in the HapA26–525 background (570–572/AAA, 588–590/AAA, 640–642/AAA and 653–656/AA) and then expressed the mutant derivatives in strain DB117. Mutation of these residues had minimal effect on the amount of Hap in the outer membrane (OM) (Fig. 4b). As shown in Fig. 4(b), assessment of adherence to full-length fibronectin immobilized on 96-well plates revealed decreased binding by strains expressing HapETD570AAA, HapTED588AAA and HapEN655AA, but not HapEIV640AAA as compared with HapA26–525, although none of these mutations completely eliminated adherence.

To ascertain whether combining the mutations would eliminate binding, we made double acidic motif mutations and tested the ability of these mutant derivatives to bind to fibronectin. As shown in Fig. 4(c), combining the mutations did not further decrease adherence to fibronectin, indicating that there may be additional Hap residues involved in this interaction. Finally, a triple acidic motif mutation (ETD570AAA, TED588AAA and EN655AA) also did not eliminate binding to fibronectin, despite the fact that the amount of Hap in the outer membrane was decreased in this strain compared with the parental strain (Fig. 4c).
The secondary structure of the acidic motifs in many Gram-positive bacterial fibronectin binding proteins is thought to be influenced by double glycine residues that are commonly found downstream of the acidic motifs (Bingham et al., 2008). The 588–590/TED and 655–656/EN motifs that we targeted were closely followed by double glycines at amino acids 597–598 and 661–662 (Fig. 4a). As shown in Fig. 3, these double glycines are surface-exposed but are located on the opposite face of Hap relative to the acidic motifs. To assess the role of the double glycines in Hap binding to fibronectin, we mutated the glycine residues to threonines in the HapΔ26–525 background (GG597TT and GG661TT) and tested the ability of these mutants to bind to fibronectin. As shown in Fig. 4(d), these mutations were associated with similar amounts of Hap in the outer membrane compared with the parental strain and resulted in reduced Hap-mediated bacterial binding to fibronectin. The double glycine mutations were also made in concert with their corresponding acidic motifs. These additional mutations did not eliminate binding to fibronectin, again suggesting that multiple points on the Hap protein are involved in binding to fibronectin.

**Disruption of Hap–Hap interactions does not affect fibronectin binding**

Amino acids 726–1036 of the Hap passenger domain are required for Hap–Hap interactions that lead to microcolony formation (Fink et al., 2003). The HapS crystal structure reveals an ordered prism made of multiple β-loops that is essential for Hap multimerization (Meng et al., 2011). Because a large region of the Hap passenger domain seems to be involved in fibronectin binding, we wondered if Hap multimerization was necessary to create a binding surface for fibronectin. We utilized HapS243A constructs that lack protease function and, therefore, remain on the bacterial surface with deletions in the prism of two and four β-loops (Δ751–789 and Δ751–827) in binding assays with full-length fibronectin immobilized on 96-well plates. As shown in Fig. 5, bacteria expressing the loop mutant constructs adhered to fibronectin at wild-type levels, suggesting that Hap–Hap interactions do not have a role in fibronectin binding.

**DISCUSSION**

*H. influenzae* is known to preferentially adhere to damaged epithelium where ECM proteins or basement membrane may be exposed, and the Hap passenger domain (HapS) is known to bind to the ECM proteins fibronectin, laminin and collagen IV (Fink et al., 2002; Read et al., 1991, 1992). In this study, we investigated the interaction between HapS and fibronectin and found that the fibronectin FNIII(1–2) repeats are necessary for Hap-mediated binding. Additionally, we observed that multiple mutations in the Hap ECM binding domain resulted in decreased bacterial binding to fibronectin.

These mutations did not abolish binding, suggesting that multiple Hap regions interact with fibronectin.

In earlier work, we found that HapS binds to the gelatin-binding domain but not the heparin-binding domain in fibronectin (Fink et al., 2002). In this report, Far-Western dot blot assays using purified HapS and a defined set of fibronectin fragments showed that HapS specifically binds to the fibronectin fragment FNIII(1–2). HapS was not able to bind to fibronectin fragments lacking the FNIII(1) repeat such as FNIII(2–3), FNIII(2–5) or FNIII(3–5), indicating either that HapS binds only to FNIII(1) or that HapS binds to both FNIII(1) and FNIII(2) together. To distinguish between these two possibilities, we studied purified HapS and Hap-mediated bacterial binding to the individual repeat units and found that binding did not occur unless FNIII(1) and FNIII(2) were together in a single fragment. Indeed, others have shown thatFNIII(2) stabilizes FNIII(1) and may protect its C-terminal end from unfolding (Oberhauser et al., 2002; Vakonakis et al., 2007). It is possible thatFNIII(2) is also required to keep FNIII(1) in the proper conformation for Hap binding. Interestingly, in the current study Hap did not bind to FNI(1–9)II(1–2), which corresponds to the gelatin-binding domain, raising the possibility that the commercially available gelatin-binding domain of fibronectin in our earlier work was contaminated with other fragments containing FNIII(1–2) (Fig. 1b).

The fibronectin region spanning FNIII(1–2) is involved in important cellular interactions (Ohashi & Erickson, 2005; Peters & Mosher, 1987; Sottile & Wiley, 1994; Vakonakis et al., 2007). Because antibodies that recognize FNIII(1)
inhibit matrix assembly (Pankov & Yamada, 2002), the interaction of HapS with the FNIII(1–2) repeats may indicate that Hap is involved in a destabilization of the fibronectin matrix. Destabilization of the matrix could aid in the spread of H. influenzae through the submucosa to the basement membrane, creating a protective niche for the organism and facilitating persistence.

Fibronectin binding proteins have been characterized in a number of bacteria, including Staphylococcus aureus (FnBPA, FnBPB), Streptococcus species (Sfb, FnBA, FnBB, FSE) and Salmonella enterica Typhimurium (ShdA) (Jönsson et al., 1991; Kingsley et al., 2002; Lindgren et al., 1992, 1994; Signäs et al., 1989; Valentin-Weigand et al., 1993). A characteristic of the Gram-positive bacterial fibronectin binding proteins is the presence of multiple repeat domains (FnBRs) with conserved sequences that are required for binding to fibronectin (Westerlund & Korhonen, 1993). Alignment of the amino acid sequences from the repeat domains of these proteins reveals a requirement for highly conserved acidic-core sequences that appear to be involved in the interaction between the binding protein and fibronectin (Bingham et al., 2008). Two glycine residues adjacent to the acidic core are also conserved and are thought to serve a structural role for the acidic motifs (Westerlund & Korhonen, 1993). A search for similar motifs in the putative ECM binding domain of HapS revealed four similar acidic sequences, two of which have nearby double glycines. In our experiments involving mutation of these acidic sequences, isolated mutations at three of the four sites resulted in a 40–60% reduction in binding to full-length fibronectin. However, when the mutations were combined, binding to fibronectin was slightly decreased beyond the isolated mutations but was not completely eliminated. These results suggest that flanking residues beyond the acidic core residues may be involved in the HapS interaction with fibronectin as well. Mutation of the double glycine residues also decreased Hap-mediated binding to fibronectin, but again binding was not totally abolished.

In previous work, we found that the C-terminal region of HapS forms a prism-like structure that gives rise to three unique faces, designated F1, F2 and F3 (Fig. 3b) (Meng et al., 2011). The EIV, VVS, TED and GG597 motifs are located on the F3 face, while EN, ETD and GG661 are located on the F1 face. There are approximately 30 residues between FNIII(4) and FNIII(2), extending >110 Å and potentially allowing the fibronectin molecule to fit around HapS and interact with the F1 and F3 faces. In conclusion, this study has provided insights into the pathogenesis of H. influenzae disease by characterizing the interaction between Hap and fibronectin. Hap-mediated adherence to ECM proteins like fibronectin may contribute to H. influenzae colonization, persistence and spread. Hap binding to fibronectin appears to be a complex process involving a larger region of the HapS passenger domain than a simple binding pocket, similar to other fibronectin binding proteins. Further exploration into the Hap binding region and comparison with other fibronectin binding proteins will increase our knowledge of the interaction between pathogenic bacteria and the ECM, an important process in bacterial pathogenicity.

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