A fine-tuned interaction between the trimeric autotransporter Haemophilus surface fibrils and vitronectin leads to serum resistance and adherence to respiratory epithelial cells

Birendra Singh, Yu-Ching Su, Tamim Al-Jubair, Oindrilla Mukherjee, Teresia Hallström, Matthias Mörgelin, Anna M. Blom, and Kristian Riesbeck

Medical Microbiology, Department of Laboratory Medicine Malmö, Lund University, Jan Waldenströms gata 59, SE-205 02 Malmö, Sweden, Department of Infection Biology, Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knoell-Institute, D-07745 Germany, Section of Clinical and Experimental Infectious Medicine, Department of Clinical Sciences, Lund University, SE-221 84 Lund, Sweden, and Division of Medical Protein Chemistry, The Wallenberg Laboratory, Department of Laboratory Medicine Malmö, Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden.

*Address correspondence and reprint requests to Dr. Kristian Riesbeck, Medical Microbiology, Department of Laboratory Medicine Malmö, Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden. E-mail: kristian.riesbeck@med.lu.se

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Abbreviations used in this article: BD, binding domain; COPD, chronic obstructive pulmonary disease; ECM, extracellular matrix; FH, Factor H; Hib, Haemophilus influenzae type b; NTHi, non-typable Haemophilus influenzae; PD, putative domain; TAA, trimeric autotransporter adhesin; VDS, vitronectin-depleted serum; Vn, vitronectin
Abstract

*Haemophilus influenzae* type b (Hib) escapes the host immune system by recruitment of the complement regulator vitronectin that inhibits the formation of the membrane attack complex (MAC) by inhibiting C5b-C7 complex formation and C9 polymerization. We previously reported that Hib acquires vitronectin at the surface by using *Haemophilus* surface fibrils (Hsf). Here we studied in detail the interaction between Hsf and vitronectin and its role in inhibition of MAC formation and invasion of lung epithelial cells. The vitronectin-binding region of Hsf was defined at the N-terminal comprising amino acids Hsf 429-652. Moreover, the Hsf recognition site on vitronectin consisted of the C-terminal amino acids 352-374. *H. influenzae* was killed more rapidly in vitronectin-depleted serum when compared to normal human serum (NHS), and an increased MAC deposition was observed at the surface of an Hsf-deficient *H. influenzae* mutant. In parallel, Hsf-expressing *E. coli* selectively acquired vitronectin from serum that resulted in significant inhibition of the MAC. Moreover, when vitronectin was bound to Hsf an increased bacterial adherence and internalization of epithelial cells was observed. Taken together, we have defined a fine-tuned protein-protein interaction between Hsf and vitronectin that may contribute to an increased virulence of Hib.
Introduction

*Haemophilus influenzae* is a Gram-negative respiratory pathogen. The species are divided into two groups depending upon the presence or absence of a polysaccharide capsule. Encapsulated strains are further divided into six different serotypes a, b, c, d, e, and f, whereas unencapsulated strains are also referred to as nontypeable *H. influenzae* (NTHi). The most virulent capsule type is the *H. influenzae* type b (Hib) that causes sepsis, pneumonia, osteomyelitis, epiglottitis, joint infections, and acute meningitis (1, 2). Although the incidence of Hib infections in developed countries has been dramatically reduced after the introduction of the Hib conjugate vaccine in the early 1990s, Hib remains a major infectious agent related to lower respiratory tract infections and causing meningitis in infants and children in many developing countries (3, 4).

The initial step of a successful colonization and subsequent infection of *Haemophilus* is bacterial adherence to host tissue, a phenomenon mediated by adhesins. Upon overcoming the mucociliary escalator, bacteria colonize and may damage the epithelial cells and break down tight junctions. Subsequently, *H. influenzae* reach the basement membrane and the extracellular matrix (ECM), and occasionally penetrate into deeper tissue layers (2, 5-7). In addition to these virulence properties, Hib often breaches the blood-brain barrier and causes inflammation of the meninges of the brain (8). The invasive mechanisms are, however, not fully understood.

*Haemophilus* surface fibrils (Hsf) is a major trimeric autotransporter adhesin initially reported in Hib. Hsf is a highly conserved protein among all typeable strains, with a monomeric size of approximately 243 kDa that builds up a trimer of approx. 750 kDa (9). In 25% of all unencapsulated strains, a homologue to the Hsf protein, *H. influenzae* adhesin (Hia) can be found. Hia is only present in approximately 25% of the clinical NTHi isolates (10), and cannot be found in encapsulated *H. influenzae*. In contrast, the Hia homologue *Haemophilus* surface
Fibril (Hsf) exists in all typeable strains (11-13). Although Hia has a smaller size of \( \approx 114 \text{ kDa} \) (\( \approx 342 \text{ kDa} \) as a trimer), these two proteins are highly homologous at their N- and C-termini with an overall 81% similarity and 72% identity. Both Hia and Hsf constitute various repetitive domains, which are also relatively similar in their secondary structures (9, 14).

Survival of Hib in the host is controlled by acquiring complement regulators to the surface of the pathogen for an effective inhibition of opsonisation, phagocytosis and formation of the membrane attack complex (MAC) (15, 16). A major regulatory component of MAC is the multifunctional glycoprotein Vitronectin (Vn) that is found both in plasma and in the ECM. It has an RGD domain in the N-terminus of the protein that is known to interact with integrins of the host epithelial cells, whereas the C-terminal heparin binding domain (HBD-3) has been shown to be used by bacterial pathogens to bind Vn (17-20). Simultaneous interaction of Vn with integrins and bacterial surface proteins has been thought to result in the formation of a bridge between bacteria and host cells. This leads to internalization as exemplified by S. pneumoniae and P. aeruginosa resulting in downstream signalling events (21-23). Protein E and Protein F of non-typeable H. influenzae has been shown to bind Vn and provide subsequent serum resistance (18, 24). Thus Vn-mediated serum resistance of Hib is multifactorial where more than one surface protein is involved in binding to the same complement regulator. In addition, H. influenzae also interacts with factor H (FH) and C4b binding protein (C4BP) for protection against complement-mediated killing (15).

We have previously demonstrated an interaction between Hsf and Vn (25). This binding was, however, not directly proven to be involved in serum resistance. In the present study, we therefore wanted to define the regions of Vn involved in the bacterial Hsf-Vn interaction, investigate the role of such interaction in serum resistance and host cell adherence. Our results
demonstrate that Vn bound to Hsf at the bacterial surface inhibits the assembly of MAC, thereby protecting the *H. influenzae* from serum-mediated killing. Moreover, Vn bound to Hsf increases the adherence and bacterial internalization of host cells.

**Materials and Methods**

**Bacterial strains and culture conditions**

The type b *H. influenzae* RM804 and its isogenic *hsf* mutant (25) were grown in brain heart infusion (BHI) liquid medium containing 10 μg ml⁻¹ nicotinamide adenine dinucleotide (NAD) and hemin, or on chocolate agar plates followed by incubation at 37 °C in a humid atmosphere with 5% CO₂. The *hsf* mutant was grown in the presence of 18 μg ml⁻¹ kanamycin. *E. coli* DH5α and *E. coli* BL21 (DE3) were cultured in Luria Bertani (LB) broth or on LB agar plates at 37 °C. *E. coli* containing expression vectors pET26b-*hsf*(s) were grown in 50 μg ml⁻¹ kanamycin and pET16b-*hsf*<sup>1-2413</sup> were supplemented with 100 μg ml⁻¹ ampicillin in LB medium. Vitronectin expressing human embryo kidney (HEK293T) cells were cultured in advanced DMEM (Gibco; Invitrogen, Stockholm, Sweden) containing 2 mM L-glutamine, 100 μg ml⁻¹ streptomycin, and 100 U ml⁻¹ penicillin.

**Vector construction, protein expression and purification**

The *sf* genes were made according to the layout described in Figure 2A. The binding domains BD<sub>2</sub><sup>429-652</sup>, BD<sub>3</sub><sup>1103-1338</sup>, and BD<sub>1</sub><sup>1792-2022</sup> were cloned. In addition to BDs, other structural Hsf motifs, putative domains (PD), that is, PD<sub>2</sub><sup>272-375</sup>, PD<sub>3</sub><sup>938-1046</sup>, and PD<sub>1</sub><sup>1637-1740</sup> (Fig. 2A) were made. Sequence specific primers were designed for PCR amplification and forward and reverse primers contained BamHI and HindIII restriction sites, respectively (Table 1). The amplified
PCR products were digested and ligated into the pET26b vector. The sequences of ligated inserts were verified from selected clones by sequencing. Finally the vectors were transformed into *E. coli* BL21(DE3). For surface expression of Hsf, pET16b-hsf<sup>1-2413</sup> and the empty vector pET16b as a control were transformed into fresh *E. coli* BL21 (DE3). Single colonies from the LB plates with ampicillin were inoculated in LB containing 100 μg ml<sup>-1</sup> ampicillin and incubated at 37°C, 200 rpm shaking. Expression was induced by addition of 0.2 mM IPTG at OD<sub>600</sub> 0.4-0.5, and cultures were grown for the next 15 h at 37°C and shaking at 200 rpm. Expression levels were verified by flow cytometry before any downstream experiment. For purification purposes, *E. coli* BL21(DE3) containing pET26b-hsf<sup>(s)</sup> was grown in LB medium with kanamycin at 37°C until OD<sub>600</sub> reached 0.8–1. Expression of the proteins was induced by addition of 1 mM IPTG. After 3 h of culture, cells were harvested and resuspended in His-tagged protein binding buffer (Tris-HCl 50 mM, pH7.5, containing 50 mM imidazole and 500 mM NaCl). Bacteria were lysed by sonication, and Ni-NTA affinity purification was performed according to the manufacturer’s guidelines (GE Healthcare Biosciences, Uppsala, Sweden). Gel filtration was performed using analytical gel filtration column (Superdex 200; GE Healthcare Biosciences), connected with AktaPrime plus FPLC system (GE Healthcare Biosciences). The column was equilibrated with PBS and approximately 200 μg of each protein fragment were injected. Separation was achieved at 0.7 ml/min at RT. Standard molecular weight markers (Sigma, St. Louis, MO) were used for comparison of molecular weights. Peaks separated by the column were collected as fractions, concentrated by centricon (5000 MWCO) and loaded on SDS-PAGE gels (Fig. S3-S4). SDS-PAGE gels were stained with Coomassie blue R250 (Fig. S1-S2). Vn constructs were expressed in HEK293T cells, as described elsewhere (18). Protein concentrations were estimated by a
Nano-drop spectrophotometer (Thermo Scientific, Wilmington, DE) and also verified by a Bicinchoninic acid (BCA) assay (Pierce, Rockford, IL).

**Western blotting**

*H. influenzae* RM804, *E. coli* control or *E. coli* expressing Hsf at the surface (10^8 cells) were blocked with PBS containing 2.5% BSA for 1 h at RT. Bacteria were resuspended in PBS containing 2.5% BSA and different serum dilutions or purified Vn were added. Binding was performed for 1 h at RT. Thereafter, bacteria were washed 3 times by PBS, resuspended in PBS and finally SDS-PAGE loading dye was added. In the following step, samples were treated at 95°C for 10 min and centrifuged at 14,000xg for 10 min. Supernatants were separated on 12% SDS polyacrylamide gels and blotted onto PVDF membranes. The membranes were blocked with PBS containing 5% milk and specific primary antibodies (AbD Serotech, Düsseldorf, Germany), anti-FH rabbit antibodies (Calbiochem, San Diego, CA), and anti-C4bp rabbit antibodies (CompTech, Tyler, TX) were added to the membrane in PBS+5% milk. After extensive washing donkey secondary antibodies conjugated with HRP were added to the membranes and developed with an ECL western blotting kit (Pierce, Rockford, IL).

**Flow cytometry**

*E. coli* cells containing pET16b-*hsf*-2413 were prepared according to the described method above. Bacteria (10^6) were resuspended in 100 µl of PBS containing 2.5% BSA and treated with affinity purified rabbit polyclonal anti-Hsf or anti-BD antibodies for 1 h at room temperature. After washing, FITC-conjugated anti-rabbit pAb (Dakopatts) were added and incubated for 1 h at room temperature. *E. coli* harboring empty vector was used as a control and stained similarly. Samples
were analyzed in a flow cytometer (EPICS XL_MCL; Beckman Coulter). MAC deposition on the surface of *H. influenzae* or Hsf-expressing *E. coli* was analyzed by flow cytometry. RM804 wild type and the Hsf-deficient mutant (RM804Δhsf) were grown in broth and washed once in PBS containing 2% BSA (PBS-BSA). Hsf-expressing *E. coli* was grown to OD$_{600}$ = 0.5 and Hsf expression was induced by 0.2 mM IPTG for 16 h. RM804 and RM804Δhsf were incubated with 5% NHS and Hsf-expressing *E. coli* and an *E. coli* control strain with 1% NHS in GVB++ buffer (CompTech) at 37°C. All bacteria were used at 10$^8$. Aliquots were removed after 5, 10, 20 and 30 min, and thereafter the bacteria were stained for MAC deposition. Bacteria were washed and incubated for 30 min with mouse anti-human MAC/C5b-9 mAb (1:100) followed by Alexa fluor 647-conjugated anti-mouse pAb (1:200). After washings, binding was analyzed by flow cytometry (FACScan LRII, Becton-Dickinson, Mountain View, CA). All incubations were kept in PBS-BSA, and primary and secondary antibodies were added separately as negative controls.

**Transmission electron microscopy (TEM)**

Anti-C9 antibodies were labelled with colloidal gold as described (26). The wild type *H. influenzae* RM804 were grown in BHI for 3 h at 37°C. *E. coli* cells were induced with IPTG to express Hsf and the expression of the protein at the surface was verified by flow cytometry prior to TEM. Bacteria were incubated with gold-conjugated antibodies, fixed in PBS containing 4% paraformaldehyde and 0.1% glutaraldehyde and prepared as described (27). TEM was performed as described elsewhere (28), and specimens were examined in a JEOL JEM 1230 transmission electron microscope (JEOL, Peabody, MA) at 60 kV accelerating voltage. The Images were recorded with a Gatan Multiscan 791 CCD camera (Gatan, Pleasanton, CA).
Direct binding assay and ELISA

A direct binding assay was used to detect $[^{125}\text{I}]$-Vn binding at the surface of bacteria. Vn was labeled by the Chloramine-T method as described (18). Approximately $1 \times 10^8 H. \text{influenzae RM804}$ or $E. \text{coli}$ were blocked with PBS-2.5% BSA and pipetted into microtiter plates. Increased concentrations of $[^{125}\text{I}]$-Vn was added to bacteria and incubated for 1 h at 37 °C. For inhibition experiments, cold ligands were added to the samples prior to addition of $[^{125}\text{I}]$-Vn. In the next step, bacteria were washed 3 times with PBS to remove unbound $[^{125}\text{I}]$-Vn. Plates were harvested in a 96-well plate harvester (Tomtec, Hamden, CT), and counted in a liquid scintillation counter (Trilux, Microbeta 1450; Perkin Elmer, Waltham, MA).

Purified Hsf fragments (50 nM) were immobilized on PolySorp microtiter plates (Nunc-Immuno, Roskilde, Denmark) in Tris-HCl, pH 9.0 for 15 h at 4°C. Plates were washed 3 times with PBS to remove unbound protein and blocked with PBS-2.5% BSA for 1 h at RT. Vitronectin was added to wells in PBS-2.5% BSA and incubated for 1 h at RT. For inhibition assays, inhibitor ligands at different concentrations were supplemented to the wells prior to addition of the Vn. The unbound Vn was removed by washing with PBS containing 0.05% Tween-20. Thereafter, the bound Vn was detected by polyclonal sheep anti-human Vn pAb and secondary horseradish peroxidase (HRP)-conjugated donkey anti-sheep pAb (both from AbD Serotec). Factor H and C4BP-binding to Hsf was also detected by ELISA by using the same protocol and specific antibodies. Finally the plates were developed and read at 450 nm in a microplate reader (Multiskan® plus, Labsystems, Helsinki, Finland).

Surface plasmon resonance (Biacore)
The interaction between Hsf and Vn was analyzed using surface plasmon resonance (Biacore 2000, Uppsala, Sweden). Four flow cells of a CM5 sensor chip were activated, each with 20 µl of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxy-sulfosuccinimide at a flow rate of 10 µl/min, after which Hsf 54-2300 and BD2 (10 µl/ml in 10 mM sodium acetate buffer, pH 4.0) were injected over separate flow cells. Unreacted groups were blocked with 20 µl of 1 M ethanolamine (pH 8.5). The final immobilization levels were 7179 resonance units (RU) for Hsf 54-2300 (244 kDa) and 884 RU for BD1 (25 kDa) and thus these were immobilized at equimolar ratios. A negative control was prepared by activating and subsequently blocking the surface of flow cell 1. Thereafter, a series of two-fold dilutions of Vn starting from 200 µg/ml were injected in the flow buffer (50 mM HEPES, pH 7.5 containing 150 mM NaCl, 3 mM EDTA and 0.005% Tween-20). Vitronectin was injected for 100 s during the association phase at a constant flow rate of 30 µl/min. The sample was first injected over the negative control surface and then over immobilized ligands. The signal from the control surface was subtracted. The dissociation was followed for 360 s at the same flow rate. In all experiments, 10 µl injections of 2 M NaCl, 100 mM HCl followed by 0.05% SDS were used to remove bound Vn during a regeneration step. The BiaEvaluation 3.0 software (Biacore) was used for data analysis using 1:1 Langmuir model of interaction.

**Serum killing assay**

Approximately 10^5 *H. influenzae* RM804 or *E. coli* expressing Hsf were resuspended in 100 µl of dextrose-GVB (DGVB++) buffer, pH 7.3, containing 140 mM glucose, 0.1 % (w/v) gelatin, 1 mM MgCl₂, 0.15 mM CaCl₂ and finally 10 % NHS (18, 24). Normal human serum was prepared from 5 different healthy volunteers according to standard guidelines. In parallel, a heat
inactivated control serum (HIS) was also prepared by inactivating complement components by heating at 56 °C for 30 min. Vitronectin-depleted serum and replenishment of new Vn in depleted serum was performed as described (24). *E. coli* expressing Hsf were optimized for their serum sensitivity by adding 1-10% NHS and HIS at different time intervals. Bacteria resuspended in DGVB++ were added with NHS/HIS and incubated at 37 °C. In a particular time interval bacteria were plated on chocolate agar. The viable bacterial cells were determined by counting colony forming units (CFU) after incubation overnight at 37 °C.

Membrane attack complex deposition assay

Microtiter plates (F96 Medisorb, Nunc-Immuno Module) were coated with Hsf or BSA, both at 5 μg/ml overnight at 4°C. The plates were washed four times with PBS-Tween and blocked for 1 h at RT with PBS containing 2% BSA. After washings, the plates were incubated for 1 h at RT with vitronectin (10-50 μg/ml) or FH (10-50 μg/ml). Subsequently, the wells were washed and incubated with C5b-6 (1.5 μg/ml) and C7 (1 μg/ml) for 10 min at RT, and thereafter C8 (0.2 μg/ml) and C9 (1 μg/ml) were added and incubated for 30 min at 37°C. MAC deposition was detected with mouse anti-human C5b-9 mAb and HRP-conjugated swine anti-mouse pAb. The reaction was developed with 1,2-phenylenediamine dihydrochloride (OPD, DakoCytomation) and the absorbance was measured at 492 nm.

H. influenzae adherence and invasion assay

Lung alveolar epithelial cells (A549) were grown in 12-well culture plates in F12 medium supplemented with 10% FCS and 5 μg/ml gentamicin. Thereafter, 80-90% confluent cells were
kept overnight in serum free media (F12 only), prior to the experiments. *H. influenzae* (10⁷ cfu) were added to cells and incubated at 37°C with gentle shaking (50 rpm). At different time points (1-6 h), cells were washed thoroughly and cells were taken out by treating with Accutase (Life Technologies, Stockholm, Sweden). Cells were lysed by glass beads and vortexing. To analyze the internalized bacteria, cells were treated with gentamicin (100 μg/ml) for 1 h to kill extracellular bacteria. Finally, different dilutions were made and solutions were spread on chocolate agar plates. To observe an effect of Vn, cell monolayers were treated with Vn (2-4 μg/ml) at 4°C for 2 h followed by extensive washing (3 times) with PBS. The additional steps were performed as described above.

**Confocal microscopy**

A549 cells were grown on glass coverslips in F12 medium supplemented with 10% FCS and 5 μg/ml gentamicin. Confluent cells (90%) were kept in serum free medium (F12 only) overnight. Bacteria were labelled with FITC (10 μg/ml) in PBS for 30 min at RT, followed by 3 washes to remove unbound FITC. The FITC-conjugated bacteria (10⁸) were added to the cells and incubated for 2 to 8 h at 37°C with gentle shaking. Cell monolayers were treated with 10 μg Vn and incubated for 2 h at 4°C, prior to addition of bacteria. Coverslips were washed 3 times in PBS and fixed by using 4% paraformaldehyde. Finally, coverslips were mounted with Vectashield (Vector Laboratories, UK) containing DAPI. The images were taken using a confocal microscope (Carl Zeiss 710, Gottingen, Germany) and processed using Zen Software.
Statistics

One- or two-way ANOVA tests were used to compare the difference between more than two experimental groups when indicated. Differences were considered statistically significant at $p \leq 0.05$. Statistical analyses were performed using GraphPad Prism® version 5.0 (GraphPad Software, La Jolla, CA).
Results

Haemophilus influenzae type b (Hib) clinical isolates bind Vn

To prove that Vn-binding to Hib strains is a common phenomenon, we analyzed Hib clinical isolates \(n=26\) for their Vn-binding capacity. Out of 26 isolates, 11 and 10 Hib had a high and medium Vn-binding capacity, respectively. *E. coli* DH5α did not bind Vn and was included as a negative control in our assay (Fig. 1A). In parallel, the Vn-binding capacity of RM804 Δhsf (25) was tested, and when compared to the clinical isolates, 19.2% of these had a similar lower Vn-binding capacity as the RM804 Δhsf mutant (Fig. 1B). We thus defined this group of clinical isolates as low Vn binders. Moreover, when Hsf was expressed at the surface of the heterologous host *E. coli*, a significant Vn-binding was observed in comparison to *E. coli* containing an empty vector only (Fig. 1C). Taken together, our data suggested that most of the clinical Hib isolates had the capacity to acquire Vn.

The binding domain-2 of Hsf recognizes vitronectin

To define the Vn-binding region in the Hsf molecule, we expressed full length Hsf^{54-2300} and the truncated fragments comprising Hsf^{54-608}, Hsf^{608-1351}, Hsf^{1047-1751}, Hsf^{1536-2031}, and Hsf^{1755-2313} (Fig. 2A). On the basis of previously identified epithelial cell binding domains, BD2 (Hsf^{429-652}), BD3 (Hsf^{1103-1338}), and finally BD1 (Hsf^{1792-2022}) were expressed in *E. coli* (9). In addition to these BDs, we also observed a set of repeats present in the putative domain (PD) 2 (Hsf^{265-376}), PD3 (Hsf^{322-1047}), PD1 (Hsf^{4621-1741}) that share 65.8-80.2% sequence similarity. The function of those repeats is at present unknown, and therefore we named them as PDs. Hence, recombinant PD1, 2, and 3 were also included in our study (Fig. 2A). The purified proteins were verified for...
their oligomeric conditions by using gel filtration and subsequent SDS-PAGE of separated protein peaks. All purified proteins were mainly trimers, with minor fractions of higher molecular weight oligomers (Fig. S1-S2). Results with ELISA showed that recombinant \( Vn^{80-396} \) significantly bound to \( Hsf^{64-2300} \), \( Hsf^{64-608} \), BD2, and \( Hsf^{536-2031} \) (Fig. 2B). Low level interactions were also observed with the PD2, BD1, and \( Hsf^{1047-1751} \) fragments. The remaining fragments (PD3, PD1, BD3, \( Hsf^{608-1351} \), and \( Hsf^{1047-1751} \)) did not interact with \( Vn \). A truncated \( Moraxella catarrhalis \) IgD binding protein (MID; amino acids 962-1200) was used as a negative control (Fig. 2B). The non-Vn-binding Hsf fragments were consequently excluded from the following downstream experiments.

To confirm the interaction with the Vn-binding Hsf fragments, we also did an inhibition assay. Vintronectin\(^{80-396}\) in solution was pre-incubated with increasing concentrations of truncated Hsf fragments (Fig. 2C) prior to addition to the full length \( Hsf^{64-2300} \). The results with recombinant \( Hsf^{64-2300} \), BD2 and \( Hsf^{64-608} \) showed a significant dose-dependent inhibition of \( Vn^{80-396} \) binding to \( Hsf^{64-2300} \). The remaining proteins (PD2, BD1, \( Hsf^{1047-1751} \) and \( Hsf^{536-2031} \)), which all showed only a minor binding to \( Vn \) (Fig. 2B) did not inhibit the \( Hsf^{64-2300} \)-interaction with \( Vn \) (Fig. 2C). As expected, heparin inhibited Vn-binding to Hsf and was included as a positive control (Fig. 2C). Since PD2 and BD1 were unable to block the interaction, these two domains were excluded as possible binding partners. Furthermore, the C-terminal region of \( Hsf^{536-2031} \) did not inhibit Vn-binding to \( Hsf^{64-2300} \). Our results clearly suggested that BD2, which is also present in \( Hsf^{54-608} \), was the major Vn-binding region of the Hsf molecule.

The binding affinity of the \( Hsf^{64-2300} \) and BD2 interaction was measured using surface plasmon resonance. Recombinant Hsf was coated on a CM5 sensor chip and binding of several concentrations of \( Vn^{80-396} \) was measured in real time. \( Vn^{80-396} \) bound \( Hsf^{64-2300} \) with \( K_{\text{ass}}=4.5 \times 10^4 \).
1/Ms, $K_{\text{dis}}=2.69 \times 10^{-4}$ 1/s and the calculated dissociation constant was $K_D=1.39 \times 10^{-8}$ M (Fig. 2D). Similarly, Vn$^{80-396}$ bound BD2 with $K_{\text{ass}}=5.3 \times 10^{4}$ 1/Ms, $K_{\text{dis}}=5.9 \times 10^{-4}$ 1/s and the dissociation constant was estimated to $K_D=1.58 \times 10^{-8}$ M (Fig. 2E). Other fragments did not significantly interact with Vn$^{80-396}$ (data not shown). Our results thus showed that BD2 and the full length Hsf interacted with Vn$^{80-396}$ with the same binding affinity.

Vn$^{352-374}$ serves as the Hsf interaction site

We previously reported that H. influenzae proteins E (18) and F (24) interact with the heparin binding domain (HBD3) at the C-terminal part of the Vn molecule. Here we observed that heparin also blocked the interaction between Hsf and Vn (Fig. 2C). Three different constructs encompassing HBD3 (Vn$^{352-362}$, Vn$^{362-374}$, and Vn$^{352-374}$) were therefore used to analyze binding to Hsf (Fig. 3A). Recombinantly expressed Vn fragments were verified for their purity and oligomeric forms by using SDS-PAGE and western blot (Fig. S3). Since serum Vn exists in both monomeric and multimeric forms we analyzed our purified Vn fragments. These consisted of mainly a multimeric population as demonstrated in Fig. S3. Some pathogens, e.g., S. pneumoniae (22) and N. meningitides (29) preferentially interact with the multimeric form of Vn. We thus tested both monomeric and multimeric Vn with Hsf$^{54-2300}$ and found that multimeric Vn had a 15-20% higher Hsf-binding capacity (Fig. S3).

To determine the binding of Vn variants to H. influenzae RM804 and Hsf-expressing E. coli (Fig. S4), bacteria were incubated with Vn$^{80-396}$, Vn$^{352-362}$, Vn$^{362-374}$ or Vn$^{352-374}$. The unbound proteins were removed by extensive washing, and fractions bound to Hsf were analyzed by western blotting. Vn$^{80-396}$, Vn$^{352-362}$ and Vn$^{362-374}$ interacted with H. influenzae RM804, whereas deletion of 20 aa at Hsf$^{352-374}$ completely abolished binding to bacteria (Fig. 3B).
Binding of Vn variants was also verified by using a direct binding assay, where Vn\textsuperscript{80-396} was labeled with radioactive iodine. Interestingly, [\textsuperscript{125}I]-Vn\textsuperscript{80-396} bound to Hsf-expressing \textit{E. coli} in a dose-dependent manner with $K_D=69.5 \times 10^{-9}$ M and $B_{\text{max}}=15.92 \times 10^{-9}$ M, whereas the \textit{E. coli} control did not interact with [\textsuperscript{125}I]-Vn\textsuperscript{80-396} (Fig. 3C-D). The binding specificity of the Vn variants was verified by a competition assay in the next set of experiments. \textit{H. influenzae} RM804 and the Hsf-expressing \textit{E. coli} were incubated with Vn\textsuperscript{80-396}, Vn\textsuperscript{Δ352-362}, Vn\textsuperscript{Δ362-374}, and Vn\textsuperscript{Δ352-374} before addition of [\textsuperscript{125}I]-Vn\textsuperscript{80-396}. The recombinant Vn\textsuperscript{80-396}, Vn\textsuperscript{Δ352-362} and Vn\textsuperscript{Δ362-374} significantly blocked the interaction of [\textsuperscript{125}I]-Vn\textsuperscript{80-396} to Hib and Hsf-expressing \textit{E. coli} (Fig. 3E).

However, the deletion mutant Vn\textsuperscript{Δ352-374} did not inhibit binding of [\textsuperscript{125}I]-Vn\textsuperscript{80-396} to bacteria.

Our binding studies of the Hsf-Vn interaction were further confirmed by analyzing protein-protein interactions in ELISA. Hsf\textsuperscript{54-2300} was coated in microtiter plates, and increasing concentrations of Vn\textsuperscript{80-396}, Vn\textsuperscript{Δ352-362}, Vn\textsuperscript{Δ362-374} or Vn\textsuperscript{Δ352-374} were added. Bound protein fractions were determined by an anti-Vn pAb. Interestingly, Vn\textsuperscript{80-396}, Vn\textsuperscript{Δ352-362} and Vn\textsuperscript{Δ362-374} bound Hsf\textsuperscript{54-2300} significantly higher when compared to Vn\textsuperscript{Δ352-374} (Fig. 3F). In conclusion, our results indicated that the C-terminal region of Vn consisting of amino acids 352-372 is necessary for the interaction with \textit{H. influenzae} RM804 and the Hsf molecule.

\textbf{Hsf acquires Vn from serum and contributes to serum resistance}

We have previously reported that \textit{H. influenzae} RM804 binds Vn, and that the isogenic \textit{H. influenzae} RM804 Δhsf mutant binds significantly less Vn (25). These analyses were performed with purified human Vn from Sigma. In the current study, we used a heterologous \textit{E. coli} system in parallel to \textit{H. influenzae} for proving the fine tuned Hsf-Vn interaction, and furthermore
analyzed the role of Vn in serum resistance. Hsf-expressing *E. coli* (Fig. S4) bound recombinant 
$[^{125}\text{I}]-\text{Vn}^{80-396}$ in a dose-dependent manner with $K_D = 69.5 \times 10^{-9}$ M (Fig. 3C-D). The Hsf-
expressing *E. coli* had a significantly increased serum resistance in comparison to the *E. coli*
control that harbored an empty plasmid (Fig. 4A, Fig. S5A). In agreement with previously
published data (25), we also showed that *H. influenzae* Δhsf had a significantly reduced serum
resistance in comparison to the Hsf-expressing wild type (Fig. S5B).

To load bacteria with complement regulatory serum factors, we pre-incubated Hsf-
expressing *E. coli* and the *E. coli* control with various concentrations of human heat-inactivated
serum (HIS). This step allowed bacteria to retain the serum proteins at their surface without
being killed. After washing to remove unbound serum components, bacteria were resuspended in
1% NHS. After 5 min of incubation at 37°C, CFU were determined by plating the bacteria. Hsf-
expressing *E. coli* were significantly more serum resistant when pre-treated with 2.0-10% HIS,
compared to control *E. coli* devoid of Hsf that did not show any increased serum resistance with
or without HIS pre-treatment (Fig. 4B). Hsf-expressing *E. coli* that was pre-incubated with HIS
(Fig. 4B) were further analyzed for deposition of complement regulators at their surface by
western blotting. Only binding of Vn was detected, whereas binding of the complement
regulators C4BP and FH was not observed (Fig. 4C). In parallel, purified serum proteins Vn,
C4BP, and FH were also tested for their interaction with Hsf-expressing *E. coli*. In contrast to the
wild type *E. coli*, only Hsf-expressing *E. coli* bound Vn (Fig. 4C, lower panel). The Hsf-Vn
interaction was also confirmed at the protein-protein interaction level. When Hsf$^{54-2300}$ was
coated on a microtiter plate and 10 nM of purified Vn, FH or C4BP were added, we found that
only Vn interacted with Hsf (Fig. 4D). Taken together, our results indicated that a complement
regulatory factor acquired by Hsf-expressing *E. coli* protected bacteria from the bactericidal activity of serum. Importantly, this serum factor was proven as Vn (Fig. 4C-D).

**H. influenzae** *is protected from serum-mediated killing when Vn is available in serum*

To evaluate the role of the Hsf-mediated Vn-interaction in inhibition of the terminal complement pathway, we prepared a Vn-depleted serum (VDS) as previously described (24). When *H. influenzae* RM804 and the corresponding Hsf-mutant *H. influenzae Δhsf* were incubated with the VDS, *H. influenzae* RM804 revealed a significantly decreased (55-60%) serum survival in comparison to NHS (Fig. 5A and S5B). In contrast, *H. influenzae Δhsf* did not show any significant difference in survival in either NHS or VDS (Fig. 5A). To further confirm the role of Vn, we supplemented Vn<sup>20-396</sup> (36-180 nM) to VDS, and incubated *H. influenzae* RM804 with this replenished serum. Supplementation of Vn (36 or 72 nM) to the VDS caused a significant increase of serum resistance, while this was not observed with mutant *H. influenzae Δhsf* (Fig. 5A).

In parallel to the experiments with *H. influenzae*, *E. coli* expressing Hsf were also incubated with NHS and VDS. Results obtained at 5 min incubation showed a reduced serum resistance in VDS in comparison to NHS (Fig. 5B), whereas no difference was observed in VDS after 5 min (Fig. S4C). To confirm the role of Vn, VDS was also supplemented with Vn<sup>20-396</sup>. Incubation of Hsf-expressing *E. coli* with VDS at 72 and 180 nM Vn<sup>20-396</sup> had a significantly increased survival in comparison to the *E. coli* control and VDS only (Fig. 5B). Taken together, our results with Hsf-expressing Hib or *E. coli* clearly indicated that bacteria recruit Vn from NHS that inhibits the complement-mediated activity and thus enhances bacterial survival.
Hsf-mediated vitronectin-binding results in decreased MAC deposition

To demonstrate that vitronectin bound to Hsf was functionally active, we determined MAC deposition in the presence of vitronectin bound to recombinant Hsf using purified components of the terminal pathway. The human C3 convertase regulator FH was used in parallel as a negative control. As can be seen in Fig. 6A, the binding of Vn was confirmed by ELISA. Vn or FH was added to wells coated with Hsf followed by addition of C5b-6 C7, C8 and C9. Deposition of MAC was determined by using specific mouse anti-C5b-9 mAbs. Vn (50 µg/ml) inhibited formation of the MAC by 34% when compared to samples without Vn added (Fig. 6C). Addition of 60-300 nM (10-50 µg/ml) FH showed a minor interaction with Hsf (Fig. 6B). This interaction was not observed when we added 10 nM FH to the Hsf-coated wells (Fig. 4D). In contrast, MAC deposition was not inhibited when FH was supplemented (Fig. 6C). These results clearly showed that Vn bound to Hsf was functionally active and inhibited MAC formation and deposition.

The level of serum resistance of *H. influenzae* RM804 and the corresponding Hsf-deficient mutant *H. influenzae* RM804Δhsf was confirmed by measurement of MAC deposition at the bacterial surface. Bacteria were incubated with NHS followed by analysis of MAC deposition at different time points using specific anti-C5b-9 antibodies and flow cytometry. A significantly lower deposition of MAC was seen on the surface of *H. influenzae* RM804 in comparison with the Hsf-deficient mutant (Fig. 6D). In addition, *H. influenzae* RM804 and the Hsf-deficient mutant were also analyzed in TEM after 10 min and 15 min incubation with NHS. Here, *H. influenzae* RM804 Δhsf had a markedly increased MAC deposition in comparison to the Hsf-expressing wild type *H. influenzae* RM804 (Fig. 6E). We also tested MAC deposition on our *E. coli* expressing Hsf, and found that expression of Hsf at the surface of *E. coli* inhibited MAC deposition.
deposition (Fig. 6F). Moreover, the TEM revealed that a lower amount of the MAC was

deposited on the *E. coli* surface expressing Hsf in comparison to *E. coli* transformed with an
empty vector only (Fig. 6G). Taken together, our data indicated that Vn bound at the surface of
bacteria via Hsf, was functionally active in inhibiting MAC and therefore contributed to serum
resistance.

The Hsf-dependent interaction with vitronectin increases *H. influenzae* adherence and
internalization

Vitronectin contributes to adherence and internalization of several bacterial species (21). Since it
has been shown that expression of Hsf-binding domains (BDs) at the *E. coli* surface recognise
Chang and HeLa cells (9, 30), we compared the adhesion of *H. influenzae* RM804 and Δhsf
mutants. Hsf-deficient mutants had a dramatically reduced adherence to monolayers of the type
II alveolar epithelial cell line A549 as observed by confocal microscopy (Fig. 7A). Epithelial
cells infected with *H. influenzae* RM804 WT harboured a higher number of intracellular bacteria
in comparison to cells incubated with the Hsf-deficient mutant. The adherence/internalization
capacity of *H. influenzae* RM804 and the corresponding Δhsf mutant was also quantified by
estimation of CFU at different time points. The association of the total number of bacteria with
A549 was significantly higher in the case of RM804 WT in comparison to Δhsf mutants (Fig.
7B). When internalized bacteria were estimated by a gentamicin protection assay, a clear
difference in bacterial numbers was observed between cells infected with *H. influenzae* RM804
WT or Δhsf (Fig. 7C).

To further investigate whether Vn play a role in Hsf-mediated adherence to epithelial
cells, A549 was pretreated with Vn followed by addition of bacteria. *H. influenzae* RM804 at a
higher number adhered to cells as compared to the mutant devoid of Hsf (Fig. 8A). In contrast to the Δhsf mutant, significantly enhanced adherence/invasion of RM804 WT was observed at increasing concentrations of Vn (2 and 4 μg) (Fig. 8B). A gentamicin protection assay revealed a two-fold higher intracellular bacterial number when compared to the H. influenzae Δhsf mutant (Fig. 8C). Taken together, the Hsf-dependent Vn-binding capacity of Hib promoted bacterial adherence and invasion of epithelial cells.

**Discussion**

Several respiratory pathogens recruit Vn in order to increase their survival in serum and/or to increase adherence to host cells (21). Hallström *et al.* (2006) reported that Hsf is a Vn-binding protein of Hib and showed that *hsf* deletion mutants were sensitive to the bactericidal effect of serum (25). In the same paper, Hsf$^{608-1351}$ was also reported to bind Vn that now has been corrected to Hsf$^{54-608}$ (Erratum, Hallström *et al.*, 2013). Here we further characterized the Hsf-Vn interaction and narrowed down the identified specific Vn-binding region. The results of our protein-protein interaction studies indicates that the domain BD2 (Hsf$^{429-652}$) serves as the major Vn-binding domain.

We previously reported that *M. catarrhalis* recruits Vn at the surface by using ubiquitous surface protein (Usp) A2 and that the binding region on the Vn molecule is located at Vn$^{312-396}$ (17). Similarly, we also published that *S. pneumoniae* PspC interacts with amino acids 352-374 on the Vn molecule (19). Moreover, we showed that non-typeable *H. influenzae* (NTHi) binds to Vn at 353-396 amino acids by using its surface adhesin protein E (18). In a very recent study, we identified protein F (PF) as another Vn-binding NTHi protein that targets Vn$^{352-374}$ (24). Our earlier studies thus clearly indicate the presence of several bacterial surface proteins of *H.*
*H. influenzae* that recognize Vn as the same target molecule. Hsf is also known to bind Vn, but as to where on the Vn molecule Hsf binds was hitherto unknown. In agreement with the previous findings with other pathogens such as *Moraxella* and pneumococci, our present study show that Hib Hsf also targets Vn at the region 352-374.

The structure and function of Hsf is still not very well defined. It has been reported that the BD1 and BD2 function as adhesive domains and bind to Chang conjunctival epithelial cells *in vitro*. These binding domains of Hsf have previously been modeled by using binding domains of Hia as a template. The same study showed that mutating D1935E of BD1 resulted in loss of Hsf-dependent interactions with epithelial cells. Homology modeling of BD2 suggested that E569 played a role in the binding pocket. When E569 was mutated to D569, the BD2 domain also reported a marked increased adhesive capacity to epithelial cells (30). We confirmed that the BD2 domain of Hsf has adhesive and Vn-binding properties supporting previous findings.

Our data supports the idea of Hsf functioning as an adhesin. It is, however, unclear whether Vn is a direct receptor for Hsf at the surface of the epithelium. In this study, Hsf-deficient mutants had a decreased adherence and internalization in the absence of Vn (Fig. 7). In contrast, the presence of Vn revealed an enhanced adherence/internalization of *H. influenzae* RM804 WT compared to the Hsf-deficient mutant counterpart (Fig. 8). It has been shown that the Hsf BD1 binds to epithelial cells more efficiently in comparison to BD2 (30). We speculate that BD1 might be a privileged region that interacts with presently unknown host epithelial surface receptors. In contrast to BD1, BD2 function as a major Vn-binding region. Upon binding to the Hsf BD2, Vn would then function as a bridge molecule between bacteria and epithelial cell surface integrins. It is also possible that BD1 may directly bind to receptors on the host.
epithelium. This may lead to a stronger Hsf-mediated "dual" interaction of *H. influenzae* RM804 with the host cell surface.

It is a well-known fact that the complement system is activated via the classical, alternative, and lectin mediated pathways (31), and is governed, amongst others, by the three regulators, C4b binding protein, FH, and Vn. While complement inhibitors such as FH and C4b-binding protein inhibit the alternative pathway or the classical/lectin-mediated pathways, respectively, Vn inhibits the assembly of the membrane binding site of the C5b7 complex and polymerization of C9 during formation of the lytic pore. Thus Vn would be an effective regulator that would inhibit the terminal lytic pathway regardless of whichever complement pathway that has been activated previously. Our current data indicate that Hsf preferentially bound to Vn and utilized it for enhancement of the resistance against the MAC.

In conclusion, our present study shows that the previously reported Hsf-Vn binding is directly involved in protecting Hib from serum-mediated killing through inhibition of the MAC complex. The results also demonstrate how Hsf might act as an adhesin by binding to Vn which in turn promotes adherence and internalization of the pathogen. Taken together, our study sheds light upon how Hib utilizes the multifunctional host protein Vn for evasion of the immune system and efficient survival in the host.

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References


|---|---|


**Figure legends**

**FIG 1** *H. influenzae* type b (Hib) clinical isolates significantly bind Vn. 

A. Direct binding of $[^{125}\text{I}]-\text{Vn}$ to clinical isolates. 

*E. coli* DH5α was used as a negative control. 

B. *H. influenzae* RM804 Δhsf showed a decreased Vn-binding in comparison to the Hsf-expressing wild type. 

C. *E. coli* expressing Hsf revealed a Vn-binding phenotype.

**FIG 2** BD2 is the major Vn binding region of Hsf. 

A. Schematic cartoon of Hsf showing a plan of the different recombinantly expressed fragments. 

B. Hsf fragments (50 nM) were coated on ELISA plate and Vn$^{80-396}$ (20 nM) was added to each well, the unbound fraction was removed by PBST and bound Vn was detected with anti-Vn sheep pAb and HRP-conjugated anti-sheep donkey pAb. 

C. Results obtained by ELISA on inhibition of the Vn$^{80-396}$ interaction with Hsf$^{54-2300}$. Increasing concentrations of recombinant Hsf fragments were incubated with Vn$^{80-396}$ prior to addition of Vn to wells coated with Hsf$^{54-2300}$. Heparin was used as a positive control. 

Statistical analyses for all data were performed by a two-way ANOVA. All data represent the mean of three independent experiments and error bars show standard deviations (SD). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. 

D. Surface plasmon resonance (Biacore) showing Vn-binding to Hsf$^{54-2300}$ that was immobilized on the chip. 

E. BD2 was immobilized and Vn-binding was analyzed. Hsf$^{54-2300}$ and BD2 were immobilized and Vn at increasing concentrations was injected. Sensorgrams (black lines) are shown. The obtained binding curves were analyzed using 1:1 Langmuir model to obtain kinetic parameters, and the resulting curves are shown in red. 

BD: binding domain, PD: putative domain.
FIG 3  Vitronectin amino acids 352-374 bind to the Hsf molecule. A. A cartoon that delineates the Vn molecule and Vn fragments including the deletion mutants. B. *H. influenzae* RM804 and Hsf-expressing *E. coli* were incubated with the recombinant proteins Vn\(^{\Delta352-362}\), Vn\(^{\Delta362-374}\) and Vn\(^{\Delta352-374}\). Vn bound to bacteria was detected using western blotting with anti-Vn antibodies. *E. coli* containing an empty pET16b vector was included as a negative control. C. A direct binding assay that demonstrates the \([^{125}\text{I}]\)-Vn\(^{80-396}\) interaction with Hsf-expressing *E. coli* and the *E. coli* control. Statistical analyses were done by the one-way ANOVA. D. Curve fitting of the \([^{125}\text{I}]\)-Vn\(^{80-396}\) binding to *E. coli* expressing Hsf to calculate Bmax and K\(_D\) values. The small insert represents scatchard plot prepared from the data. E. Inhibition of \([^{125}\text{I}]\)-Vn\(^{80-396}\) binding to *H. influenzae* RM804, and Hsf-expressing *E. coli* by cold Vn\(^{\Delta352-362}\), Vn\(^{\Delta362-374}\) and Vn\(^{\Delta352-374}\) (all at 0.5 µM). F. Binding of Vn\(^{\Delta352-362}\), Vn\(^{\Delta362-374}\) and Vn\(^{\Delta352-374}\) recombinant proteins to Hsf\(^{\text{HbD-A3}}\) by ELISA. For C, E, and F two-way ANOVA was used for statistical analyses. All data represent the mean of three independent experiments and error bars indicate SD. * P ≤ 0.05; ** P ≤ 0.01; ***P ≤ 0.001. SMB: somatomedin B, HBD: heparin binding domain, RGD: arg-gly-aspartic acid.

FIG 4  Hsf-expressing *E. coli* selectively interact with Vn and acquired serum resistance. A. Hsf expression at the surface of *E. coli* showed serum resistant phenotype. The time-dependent serum killing is shown in Figure S3. B. Hsf-expressing *E. coli* and the isogenic control were incubated with HIS followed by addition of 1% NHS. Statistical analysis was performed by the one-way ANOVA. The CFU were measured after 10 min incubation. C. *E. coli* expressing Hsf was incubated with different dilutions of normal human serum and pure Vn\(^{80-396}\), C4BP and FH. Bacteria were washed and bound fractions were detected by western blotting. This experiment was repeated twice and results of one typical blot are shown. D. Binding of 10 nM Vn, FH and
C4BP to Hsf as analyzed by ELISA. Statistical analysis was performed by a two-way ANOVA. All data represent the mean of three independent experiments and error bars indicate SD. * $P \leq 0.05$; *** $P \leq 0.001$.

**FIG 5** Vn is involved in Hsf-mediated serum resistance. A. *H. influenzae* RM804 wild type and the corresponding mutant *H. influenzae Δhsf* incubated with NHS and Vn-depleted serum (VDS). *H. influenzae* wild type and the Hsf-deficient mutant were incubated in 5% Vn-depleted serum supplemented with increasing concentrations of Vn$^{20-396}$ and incubated for 10 min. B. Hsf-expressing *E. coli* and the negative control were incubated with 1% NHS and Vn-depleted serum for 5 min. In addition, *E. coli* control and *E. coli* expressing Hsf were incubated with 1% Vn-depleted serum supplemented with increasing concentrations of Vn$^{20-396}$ and incubated for 5 min. Time dependent serum killing of A and B are shown in Figure S3. The statistical analysis was performed by using a two-way ANOVA for all panels. All data represent the mean of three independent experiments and error bars show SD. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. NHS: normal human serum, VDS: vitronectin depleted serum.

**FIG 6** Vn bound to Hsf at the bacterial surface inhibits assembly of the membrane attack complex (MAC). A-B. Microwell plates were coated with Hsf and increasing amount of Vn and FH were added to the wells. The binding was confirmed by anti-Vn and anti-FH antibodies. C. The Vn and FH coated wells were assayed for deposition of MAC complex by using anti-C5b-9 mAbs. D. Deposition of MAC at the surface of *H. influenzae* RM804 and Hsf-deficient mutants at different time points. E. TEM showing deposition of MAC at the surface of *H. influenzae*.
RM804 and the Hsf-deficient mutant at 10 and 15 min. F. Deposition of MAC at the surface of *E. coli* expressing Hsf and the *E. coli* control with an empty pET16b vector. G. TEM showing deposition of C9 at the surface of *H. influenzae* RM804 and the Hsf-deficient mutant at 10 and 15 min. The size bar represents 100 nm. All experiments (except TEM) were repeated three times. Data are presented as the mean of three independent experiments, and error bars indicate SD. * P ≤ 0.05; ** P ≤ 0.01; ***P ≤ 0.001.

**FIG 7** Hsf functions as an adhesin. A. Confocal microscopy showing adherence of RM804 WT and Δhsf mutants. Bacteria were incubated with A549 epithelial cells for 6 h and thereafter imaged. Bacteria are labelled in green (FITC) and cell nuclei in blue (DAPI). Right most panel showed merged images. The bar represents 20 μM size. B. Measurement of total bacteria associated with A549 cells by counting of CFU. C. Internalized bacteria after treatment with the extracellularly active drug gentamicin. Each well of microtiter plate have approximately 3×10^5-3.5×10^5 cells and 1×10^7 CFU were added to each well. Data are presented as the mean of three independent experiments, and error bars indicate SD. **P ≤ 0.01, ***P ≤ 0.001.

**FIG 8** Vitronectin promotes adherence and internalization of Hib. A. Confocal microscopy showing increased adherence of *H. influenzae* RM804 WT in the presence of Vn. Cell monolayers were treated with 10 μg Vn and thereafter incubated with FITC-conjugated bacteria for 2 h. Phase contrast microscopy represents whole cell images. Bacteria were labeled in green (FITC), whereas cell nuclei are blue (DAPI). Bar represents 20 μM size. B. Bacterial adherence to A549 cells after 2 h in the presence of 2 and 4 μg/ml Vn as measured by counting of CFU. C.
Internalized bacteria were measured by a gentamicin protection assay. Each well of the microtiter plates have approximately $3 \times 10^5$ - $3.5 \times 10^5$ cells and $1 \times 10^7$ bacteria CFU were added to each well. Data are presented as the mean of three independent experiments, and error bars indicate SD. ***$P \leq 0.001$. **
Table 1. List of primers used for cloning of recombinant protein

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A

B

C

D

E

Hsf^54-2300
K_d = 1.36 \times 10^{8} M
K_{ass} = 4.5 \times 10^{4} 1/Ms
K_{diss} = 2.69 \times 10^{4} 1/s

BD2
K_d = 1.58 \times 10^{8} M
K_{ass} = 5.3 \times 10^{4} 1/Ms
K_{diss} = 5.9 \times 10^{4} 1/s