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Haemophilus influenzae surface fibril (Hsf) is a unique twisted hairpin-like trimeric autotransporter

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ABSTRACT

The *Haemophilus* surface fibril (Hsf) is an extraordinary large (2413 amino acids) trimeric autotransporter, present in all encapsulated *Haemophilus influenzae*. It contributes to virulence by directly functioning as an adhesin. Furthermore, Hsf recruits the host factor vitronectin thereby inhibiting the host innate immune response resulting in enhanced survival in serum. Here we observed by electron microscopy that Hsf appears as an 100 nm long fibril at the bacterial surface albeit the length is approximately 200 nm according to a bioinformatics based model. To unveil this discrepancy, we denaturated Hsf at the surface of Hib by using guanidine hydrochloride (GuHCl). Partial denaturation induced in the presence of GuHCl unfolded the Hsf molecules, and resulted in an increased length of fibres in comparison to the native trimeric form. Importantly, our findings were also verified by *E. coli* expressing Hsf at its surface. In addition, a set of Hsf-specific peptide antibodies also indicated that the N-terminal of Hsf is located near the C-terminal at the base of the fibril. Taken together, our results demonstrated that Hsf is not a straight molecule but is folded and doubled over. This is the first report that provides the unique structural features of the trimeric autotransporter Hsf.

Introduction

Haemophilus influenzae is a Gram-negative respiratory pathogen that are categorised into encapsulated (serotype a to f) and unencapsulated strains, the later group is designated as non-typeable *H. influenzae* (NTHi). *H. influenzae* type b (Hib) causes pneumonia, osteomyelitis, epiglottitis, sepsis, joint infections, and acute meningitis and is hence considered as the most virulent type (Morris et al., 2008; Agrawal and Murphy, 2011). Even though the incidence of Hib infections in developed countries has been significantly reduced after introduction of the Hib conjugate vaccine in the early 1990s (Danovaro-Holliday et al., 2008), Hib remains a major infectious agent in infants and children in developing countries (Fitzwater et al., 2010). Hib infection starts by attachment of the bacteria to the nasopharyngeal and lung epithelial surfaces resulting in epithelial damage followed by penetration of the underlying tissues mediated by various sophisticated mechanisms (Geme and Cutter, 1995; Geme, 1996; Ulanova and Tsang, 2009; Agrawal and Murphy, 2011). Hib is furthermore able to penetrate the blood-brain barrier and thus causes meningitis (Singh et al., 2012). Survival of Hib in the blood is controlled by acquiring complement regulators to the surface of the pathogen for an effective inhibition of the membrane attack complex (MAC) (Winkelstein and Moxon, 1992; Hallström and Riesbeck, 2010; Singh et al., 2010). However, penetration of this pathogen to deep tissues depends on multiple virulence factors (Agrawal and Murphy, 2011).

Gram-negative pathogens possess a specific group of proteins known as autotransporters, which are translocated to the cell surface by a type V secretion mechanism. Unlike the type I–IV secretory systems that involve multiple proteins, autotransporters are composed of a single protein consisting of an N-terminal signal peptide for secretion, followed by a passenger domain, and a C-terminal translocator (membrane anchoring) domain (Dautin and Bernstein, 2007; Leo et al., 2012). These autotransporters are multifunctional proteins ranging from monomeric to multimeric arrangements (Meng et al., 2011). Recently, the biological role of few autotransporters has been studied in *H. influenzae*. The monomeric autotransporter *Haemophilus* adhesion and

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penetration protein (Hap) is involved in bacterial aggregation and adherence to host cells (Meng et al., 2011; Hallström et al., 2011). A trimeric autotransporter adhesin (TAA) known as Hia has also been extensively studied for its functional and structural characteristics (Dautin et al., 2007; Spahich and St Geme, 2011). Hia is only present in approximately 25% of the clinical NTHi isolates (St Geme et al., 1998), and cannot be found in encapsulated *H. influenzae*. In contrast, *Haemophilus* surface fibril (Hsf) is present in all typeable strains (St Geme et al., 1996; Rodriguez et al., 2003; Watson et al., 2013). Notwithstanding homologous, Hia and Hsf are relatively different sizewise; Hia has a size of ≈114 kDa (≈342 kDa as a trimer), whereas Hsf is almost twice in size with a monomer of approximately 243 kDa that builds up a ≈750 kDa trimer (Cotter et al., 2005b). These two TAAs are highly homologous at their N- and C-termini with an overall 81% similarity and 72% identity. Moreover, Hia and Hsf are constituted of various repetitive domains, which in parts have been defined according to their biological functions. For instance, Hia has two functional host epithelial cell binding domains designated BD1 and BD2. Similar binding domains are present in Hsf, but in addition, Hsf has a third binding domain (BD3). The binding domains are similar in their secondary structures. However, a few key amino acids of the binding pocket of the BD3 of Hsf are different as compared to BD1 and BD2, and therefore BD3 appears not to interact with host cells (Danovaro-Holliday et al., 2008; Spahich and St Geme, 2011).

Previously we reported that Hsf recruits Vn and thus inhibits the lytic pathway of the complement system (Hallström et al., 2006). Recently, we studied the Hsf-Vn interaction in detail and found that BD2 of Hsf selectively interacts with the C-terminal end of Vn and thus inhibits MAC formation (Singh et al., 2014). Vn also mediates an increased adherence of Hib to epithelial cells. We proposed that when Vn is bound to BD2 it may function as a bridging molecule between the bacteria and epithelial surface integrins. However, BD1 may directly bind to host epithelium, leading to a stronger Hsf-mediated interaction of Hib with the host cell surface (Singh et al., 2014).

In the present study, we investigated the molecular architecture of Hsf at the bacterial surface. Since the Hsf molecule is an extraordinary large protein and has a repetitive domain structure we modeled this protein by using an *in silico* approach. Our computed model suggested a protein length of approximately 200 nm, whereas the length of Hsf observed by electron microscopy was only 100 nm. Based upon these observations, we analyzed the organisation of Hsf on the bacterial surface by denaturing Hsf using GuHCl in order to unfold the Hsf molecule. In addition, a set of specific anti-Hsf peptide antibodies was included in the analyses to locate the precise regions of the molecule. Our results show that Hsf is not a straight fibre, as reported with several other known bacterial TAAs, but rather consists as a "hairpin-like" twisted molecule.

Material and methods

Bacterial strains and culture conditions

The type b *H. influenzae* RM804 and mutants (Hallström et al., 2006) 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. Cultures were incubated at 37 °C in a humid atmosphere containing 5% CO_2. The *hsf* mutant was grown in BHI supplemented with 18 μg ml^{-1} kanamycin. Luria Bertani (LB) broth or on LB agar plates were used to grow *E. coli* DH5α and *E. coli* BL21 (DE3). *E. coli* harboring expression vectors pET26b-*hsf*(s) and pET16b-*hsf*$^{1-2413}$ were grown in 50 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin, respectively.

Bioinformatics and protein modeling

An *in silico* model of Hsf was constructed by using a comparative protein structure modeling on domains with a highly homologous amino acid sequence to that of a known three-dimensional structure determined by X-Ray Diffraction Crystallography. Domains with low homology to known three-dimensional structures was built as a trimeric coiled-coil as described elsewhere (Griffiths et al., 2011). The initial alignment from the BLAST/PSI-BLAST homology search was refined using ClustalW alignment and prime STA alignment combined with manual alignment. From the final alignment, an energy-based homo-multimer model was constructed. The resulting model was evaluated to ensure that hydrophilic amino acids were on the surface and hydrophobic amino acids were buried in the protein interior. If these criteria were not sufficiently met, a new model was constructed after sequence re-alignment. The comparative protein structure modeling was performed using Prime version 3.1, which is part of the Schrödinger Suite 2012 (Prime, version 3.1, Schrödinger, LLC, New York, NY, 2012). In addition, UCSF Chimera 1.6 was used for bookkeeping purposes such as the renumbering of amino acid sequences (Pettersen et al., 2004).

Protein expression and purification

Recombinant DNA plasmids were obtained as described elsewhere (Singh et al., 2014). For surface expression of the Hsf protein, pET16-*hsf*54-2300 (Hallström et al., 2006) was freshly transformed into *E. coli* BL21 (DE3) and plated on LB ampicillin plates. Single colonies were inoculated in 10 ml LB containing 100 μg ml^{-1} ampicillin and incubated at 37 °C at 200 rpm shaking. Expression was induced by addition of 0.2 mM IPTG when $OD_{600\,nm}$ reached to 0.4–0.5. Induced cultures were further incubated at 37 °C with shaking at 200 rpm for the next 15 h. Expression of Hsf at the surface was verified by using anti-PD antibodies in flow cytometry. For purification purposes, *E. coli* BL21(DE3) containing pET26b-*hsf*(s) was grown in LB medium with kanamycin at 37 °C until $OD_{600\,nm}$ reached to 0.8–1. Protein expression was induced by addition of 1 mM IPTG, and protein purification was performed as described elsewhere (Singh and Rohm, 2008). Some Hsf fragments produced inclusion bodies that were purified by using an inclusion body purification protocol (Singh and Rohm, 2008). The purified proteins were dialyzed in PBS and concentrated by using Centricon cartridge (Millipore, Bedford, MA). Purity of the proteins was confirmed by SDS-PAGE and concentrations were estimated by a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and also verified by a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL).

Production of antibodies

Specific peptides covering the sequence Hsf 153–164, 876–881 (KTRAAS), 1912–1942 were synthesized and conjugated with KLH (Innovagen, Lund, Sweden). For immunization, 500 μl peptides (200 μg) were mixed with 500 μl complete Freunds adjuvant followed by immunization of rabbits. After 4 weeks, the same amount of peptides were injected with aluminium hydroxide as adjuvant. Two boosters were administered after 5 and 6 weeks. Finally, blood was drawn from immunized rabbits and antibodies (Abs) from sera were purified by standard affinity purification protocol (Singh et al., 2013). BD and PD domains were aligned and modeled to identify the conserved surface exposed regions (Fig. 1B–C and Fig. S1). Peptide antibodies against the BD and PD domains were produced in rabbits and purified by affinity purification (Genscript, NJ). Antibodies solutions were concentrated to 1 mg/ml in PBS and stored at −20 °C.