Evidence for donor strand complementation in the biogenesis of *Haemophilus influenzae* haemagglutinating pili

Graham P. Krasan,1,3 Frederic G. Sauer,2 David Cutter,1,2 Monica M. Farley,4 Janet R. Gilsdorf,5 Scott J. Hultgren2 and Joseph W. St. Geme, III1,2,3*

1Edward Mallinckrodt Department of Pediatrics and 2Department of Molecular Microbiology, Washington University School of Medicine, and 3Division of Infectious Diseases, St. Louis Children’s Hospital, St. Louis, Missouri, 63110, USA.4Veterans Affairs Medical Center and Department of Medicine, Emory University School of Medicine, Decatur, GA, 30033, USA.5Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, MI, 48109, USA.

Summary

*Haemophilus influenzae* haemagglutinating pili are surface appendages that promote attachment to host cells and facilitate respiratory tract colonization, an essential step in the pathogenesis of disease. In contrast to other well-characterized forms of pili, *H. influenzae* haemagglutinating pili are two-stranded helical structures. Nevertheless, haemagglutinating pili are assembled by a pathway that involves a periplasmic chaperone and an outer membrane usher, analogous to the prototype pathway involved in the biogenesis of *Escherichia coli* P pili. In this study, we performed site-directed mutagenesis of the *H. influenzae* HifB chaperone and HifA major pilus subunit at positions homologous to sites important for chaperone–subunit interactions and subunit oligomerization in P pili. Mutations at putative subunit binding pocket residues in HifB or at the penultimate tyrosine in HifA abolished formation of HifB–HifA periplasmic complexes, whereas mutations at the −14 glycine in HifA had no effect on HifB–HifA interactions but abrogated HifA oligomerization. To define further the constraints of the interaction between HifA and HifB, we examined the interchangeability of pilus gene cluster components from *H. influenzae* type b strain Eagan (*hifA-hifE*Eag) and the related *H. influenzae* biogroup aegyptius strain F3031 (*hifA-hifE*F3031). Functional pili were assembled both with HifA*Eag* and the strain F3031 gene cluster and with HifA*F3031* and the strain Eagan gene cluster, underscoring the flexibility of the *H. influenzae* chaperone/usher pathway in incorporating HifA subunits with significant sequence diversity. To gain additional insight into the interactive surfaces of HifA and HifB, we aligned HifA sequences from 20 different strains and then modelled the HifA structure based on the recently crystallized PapD–PapK complex. Analysis of the resulting structure revealed high levels of sequence conservation in regions predicted to interact with HifB, and maximal sequence diversity in regions potentially exposed on the surface of assembled pili. These results suggest broad applicability of structure–function relationships identified in studies of P pili, including the concepts of donor strand complementation and donor strand exchange. In addition, they provide insight into the structure of HifA and suggest a basis for antigenic variation in *H. influenzae* haemagglutinating pili.

Introduction

*Haemophilus influenzae* is a Gram-negative coccobacillus that is both a commensal organism in the human nasopharynx and an important cause of localized respiratory tract and systemic disease (Turk, 1984). Diverse isolates of *H. influenzae* express a variety of pilus and non-pilus adhesive factors (Rao et al., 1999) involved in recognition of specific eukaryotic receptor motifs (Karlsson, 1989; Hultgren et al., 1996). Perhaps best-characterized among *H. influenzae* adhesins are haemagglutinating pili, which are expressed by encapsulated and a subset of non-encapsulated (non-typable) strains (Stull et al., 1984; Gilsdorf et al., 1992; Krasan et al., 1999). These structures agglutinate AnWj-positive erythrocytes and mediate attachment to human oropharyngeal cells and respiratory tissue in organ culture (van Alphen et al., 1986; Loeb et al., 1988; Farley et al., 1990; Read et al., 1991). In addition, they facilitate nasopharyngeal colonization in monkeys (Weber et al., 1991).

Based on examination by quick-freeze, deep-etch transmission electron microscopy, *H. influenzae* haemagglutinating pili are known to be composite polymeric
structures, consisting of a two-stranded helical rod capped by a short tip fibrillum (St. Geme et al., 1996). The \textit{H. influenzae} pilus gene cluster has been cloned and sequenced from type b and non-typable strains and consists of five genes, designated \textit{hifA}–\textit{hifE} (van Ham et al. 1994; McCrea et al., 1994; 1997; Watson et al., 1994; Geluk et al., 1998; Mhlanga-Mutangadura, 1998). The \textit{hif} gene products share homology with proteins involved in the biogenesis of other pili, including P pilus produced by uropathogenic \textit{Escherichia coli} (Hultgren et al., 1996).

\textit{hifA} encodes the major structural subunit (HifA) of \textit{H. influenzae} haemagglutinating pilis and is transcribed divergently from the remainder of the gene cluster (van Ham et al., 1994). Upstream of \textit{hifA}, the \textit{hifB} gene encodes a chaperone (HifB), the \textit{hifC} gene encodes a putative outer membrane usher (HifC) and the \textit{hifD} and \textit{hifE} genes encode minor structural subunits (HifD and HifE) (van Ham et al., 1994; McCrea et al., 1994; 1997; Watson et al., 1994; St. Geme et al., 1996). Existing evidence indicates that \textit{H. influenzae} haemagglutinating pilis are assembled by the chaperone/usher pathway, originally defined in \textit{P} pilus \textit{E. coli} (Thanassi et al., 1998). HifB forms periplasmic complexes with HifA, HifD and HifE, and stabilizes these subunits, then presumably delivers them to HifC for translocation across the outer membrane (St. Geme et al., 1996). Ultimately HifA is incorporated throughout the pilus shaft, whereas HifD and HifE make up the tip fibrillum (St. Geme et al., 1996; McCrea et al., 1997). Preliminary evidence suggests that HifE is the adhesive subunit (McCrea et al., 1997).

PapD is the periplasmic chaperone involved in the assembly of \textit{P} pilis and is the prototype member of a large family of bacterial chaperones involved in interactions with pilin subunits (Holmgren et al., 1992; Hultgren et al., 1996; Hung et al., 1996). The crystal structures of PapD alone (Holmgren et al., 1992) and PapD interacting with the PapK pilin subunit (Sauer et al., 1999) have been solved. PapD consists of two immunoglobulin-like (Ig) folds oriented in an L-shape with an intervening cleft. The PapK subunit consists of a single Ig fold but lacks the expected seventh, C-terminal \(\beta\) strand (strand G). The absence of this strand leaves a deep groove along the surface of PapK and exposes its hydrophobic core, predisposing the subunit to aggregation and degradation. In the PapD–PapK complex, the G1 strand of PapD occupies the groove in PapK and completes the Ig fold, a phenomenon referred to as donor strand complementation (Choudhury et al., 1999; Sauer et al., 1999). This interaction shields the hydrophobic core of the subunit and thus stabilizes the protein. Within the groove, the G1 strand of PapD interacts on one side with the C-terminal, F strand of PapK. The Arg-8 and Lys-112 residues in the PapD cleft serve to anchor the C-terminal end of the F strand (Slonim et al., 1992; Kuehn et al., 1993; Hung et al., 1996). Mutational and biochemical studies indicate that residues in the F strand of PapK and other Pap subunits also participate in primary ‘head-to-tail’ and secondary packaging interactions between subunits in assembled pili (Bullitt et al., 1996; Soto et al., 1998). The glycine located 14 residues from the C-terminal end of subunits (−14 position) is especially important for these interactions (Bullitt et al., 1996; Soto et al., 1998).

Given the unusual two-stranded architecture of \textit{H. influenzae} haemagglutinating pilis, in the present study we performed site-directed mutagenesis of HifB and HifA to investigate the structural motifs predicted to comprise chaperone–subunit and subunit–subunit interfaces. In addition, we examined the constraints on the HifB–HifA interaction by assessing the interchangeability of pilus gene cluster components from two diverse \textit{H. influenzae} strains. Finally, we combined information from the crystal structure of the PapD–PapK complex and from HifA sequences from 20 different strains to model the structure of HifA and gain further insight into the interactive surfaces of HifB and HifA.

**Results**

\textit{HifB} and \textit{HifA} contain components of a chaperone–subunit molecular anchor motif

In the \textit{P} pilus system, the Arg-8 and Lys-112 residues in PapD cleft anchor the C-terminal end of the F strand of a given subunit and position the G1 strand of PapD relative to the F strand to complete the Ig fold of the subunit. Mutations at either Arg-8 or Lys-112 abolish chaperone–subunit complex formation, indicating the critical role of these residues in donor strand complementation (Slonim et al., 1992; Kuehn et al., 1993). To determine whether the HifB chaperone has a similar molecular anchor, we focused on \textit{H. influenzae} strain Eagan and mutated the homologous residues in the strain Eagan HifB protein.
(Arg-9 and Lys-119 respectively), replacing the Arg-9 residue with a glycine and the Lys-119 residue with an alanine (Fig. 1A). These substitutions strip the basic side chains from the original amino acids and are not predicted to cause major perturbations in protein conformation. The resulting hifB mutants were then co-expressed with pJS201 (hifAEag) in E. coli ORN103. In control experiments, we confirmed that the HifB derivatives were localized properly to the periplasm (not shown). As shown in Fig. 2A, when either pJS202.1 [HifBEag(R9G)] or pJS202.2 [HifBEag(K119A)] was co-expressed with pJS201, we were unable to detect HifA in the periplasm, consistent with periplasmic degradation.

To assess the ramifications of these binding pocket mutations on pilus biogenesis, the hifB mutants were co-expressed with pWW15ΔhifB, which contains the entire pilus gene cluster from strain Eagan with an in-frame deletion in hifB. Examination of periplasmic proteins revealed abundant HifA in ORN103/pWW15, but no detectable HifA in ORN103/pWW15ΔhifB (not shown). As expected, complementation of pWW15ΔhifB with wild-type hifBEag resulted in rescue of HifA in the periplasm and production of surface-localized pili, as measured by haemagglutination assays and whole cell dot immunoblots (Fig. 2B). In contrast, ORN103 derivatives harbouring pWW15ΔhifB together with either pJS202.1 [HifBEag(R9G)] or pJS202.2 [HifBEag(K119A)] were haemagglutination-negative and failed to react significantly with anti-HifB-HifA antiserum in whole cell dot immunoblots (Fig. 2B). The failure to produce mature pili when non-conservative residues were substituted at Arg-9 and Lys-119 underscores the importance of these positions as a molecular anchor in chaperone–subunit complex formation and pilus biogenesis.

To complement analysis of the HifB subunit binding pocket, we determined whether the C-terminal, F strand of HifA functions in donor strand complementation. An important feature of the F strand in P-pilus subunits is the penultimate aromatic residue, which interacts with the G1 strand of the chaperone. Accordingly, we targeted the tyrosine at position 215 in the strain Eagan HifA protein and examined whether mutation of this residue disrupts possible primary interactions with the chaperone (Fig. 1B). Our approach involved the generation of three separate point mutations at this site, including a conservative change that preserves the aromatic side-chain [HifAEag(Y215F)], a non-conservative substitution [HifAEag(Y215S)] and a deletion of both the penultimate and terminal residues [HifAEag(Y215Stop)]. The resulting constructs were co-expressed with pJS202 (HifBEag), and periplasmic extracts were examined by immunoblot assay with anti-HifA antiserum. As shown in Fig. 3A, mutation of Tyr-215 to phenylalanine had little effect on the quantity of HifA in the periplasm. In contrast, with HifAEag(Y215S) and HifAEag(Y215Stop), HifA was undetectable, suggesting an unstable HifB–HifA interaction.

To assess the influence of the HifA Tyr-215 residue on pilus biogenesis, the hifA mutants were co-expressed with pWW1, which contains hifB-hifEag. In control experiments, co-expression of wild-type hifAEag with pWW1 resulted in the production of functional surface-associated pili, as measured by haemagglutination assays, whole cell dot immunoblot assays and negative staining transmission electron microscopy (Fig. 3B and not shown). Consistent with our analysis of HifA levels in periplasmic extracts, co-expression of pJS206.1 [HifAEag(Y215F)] and

© 2000 Blackwell Science Ltd, Molecular Microbiology, 35, 1335–1347
pWW1 was associated with appreciable haemagglutination and significant reactivity in whole cell dot immunoblots, whereas analysis of either pJS206.2 [HifA Eug(Y215F)] or the mutant derivatives HifA Eug(Y215S) (pJS201.1) and HifA Eug(Y215Stop) (pJS201.3), probed with anti-HifA antiserum. Samples were loaded as follows: lane 1, ORN103/pJS202; lane 2, ORN103/pJS201.4 + pJS202; lane 3, ORN103/pJS201.5 + pJS202; lane 4, ORN103/pJS201.6 + pJS202; lane 5, ORN103/pJS201.7 + pJS202. The mature HifA species is indicated by an arrow.

Glycine-203 of HifA is critical for subunit–subunit interactions

In additional experiments, we found that polymerization of strain Eagan HifA subunits could be appreciated by comparing boiled and unboiled samples of periplasmic HifB–HifA complexes resolved on SDS–PAGE gels. Whereas boiling was associated with only monomeric HifA, omission of boiling resulted in a striking ladder of bands corresponding to dimers, trimers and higher order HifA multimers (Fig. 4A, compare lanes 3 boiled and unboiled). This finding complements our previous observation that purified HifB–HifA complexes serve as building blocks for spontaneous assembly of HifA subunits into pilus-like structures (St. Geme et al., 1996).

In the case of P pili, subunit–subunit interactions involve F strand residues, including the glycine at position –14 from the C-terminus (Bullitt et al., 1996; Soto et al., 1998). With this information in mind, we mutated the corresponding identical residue in the strain Eagan HifA protein (Gly-203), converting this amino acid to either unboiled). This finding complements our previous observation that purified HifB–HifA complexes serve as building blocks for spontaneous assembly of HifA subunits into pilus-like structures (St. Geme et al., 1996).

In the case of P pili, subunit–subunit interactions involve F strand residues, including the glycine at position –14 from the C-terminus (Bullitt et al., 1996; Soto et al., 1998). With this information in mind, we mutated the corresponding identical residue in the strain Eagan HifA protein (Gly-203), converting this amino acid to either

Glycine-203 of HifA is critical for subunit–subunit interactions

In additional experiments, we found that polymerization of strain Eagan HifA subunits could be appreciated by comparing boiled and unboiled samples of periplasmic HifB–HifA complexes resolved on SDS–PAGE gels. Whereas boiling was associated with only monomeric HifA, omission of boiling resulted in a striking ladder of bands corresponding to dimers, trimers and higher order HifA multimers (Fig. 4A, compare lanes 3 boiled and unboiled). This finding complements our previous observation that purified HifB–HifA complexes serve as building blocks for spontaneous assembly of HifA subunits into pilus-like structures (St. Geme et al., 1996).

In the case of P pili, subunit–subunit interactions involve F strand residues, including the glycine at position –14 from the C-terminus (Bullitt et al., 1996; Soto et al., 1998). With this information in mind, we mutated the corresponding identical residue in the strain Eagan HifA protein (Gly-203), converting this amino acid to either

Glycine-203 of HifA is critical for subunit–subunit interactions

In additional experiments, we found that polymerization of strain Eagan HifA subunits could be appreciated by comparing boiled and unboiled samples of periplasmic HifB–HifA complexes resolved on SDS–PAGE gels. Whereas boiling was associated with only monomeric HifA, omission of boiling resulted in a striking ladder of bands corresponding to dimers, trimers and higher order HifA multimers (Fig. 4A, compare lanes 3 boiled and unboiled). This finding complements our previous observation that purified HifB–HifA complexes serve as building blocks for spontaneous assembly of HifA subunits into pilus-like structures (St. Geme et al., 1996).

In the case of P pili, subunit–subunit interactions involve F strand residues, including the glycine at position –14 from the C-terminus (Bullitt et al., 1996; Soto et al., 1998). With this information in mind, we mutated the corresponding identical residue in the strain Eagan HifA protein (Gly-203), converting this amino acid to either

Glycine-203 of HifA is critical for subunit–subunit interactions

In additional experiments, we found that polymerization of strain Eagan HifA subunits could be appreciated by comparing boiled and unboiled samples of periplasmic HifB–HifA complexes resolved on SDS–PAGE gels. Whereas boiling was associated with only monomeric HifA, omission of boiling resulted in a striking ladder of bands corresponding to dimers, trimers and higher order HifA multimers (Fig. 4A, compare lanes 3 boiled and unboiled). This finding complements our previous observation that purified HifB–HifA complexes serve as building blocks for spontaneous assembly of HifA subunits into pilus-like structures (St. Geme et al., 1996).

In the case of P pili, subunit–subunit interactions involve F strand residues, including the glycine at position –14 from the C-terminus (Bullitt et al., 1996; Soto et al., 1998). With this information in mind, we mutated the corresponding identical residue in the strain Eagan HifA protein (Gly-203), converting this amino acid to either
alanine or serine. As shown in Fig. 4A, comparison of periplasmic extracts from ORN103 producing HifB and either wild-type HifAEag, HifAEag(G203A) or HifAEag(G203S) revealed no appreciable difference in the quantity of monomeric HifA present after boiling, electrophoresis and immunoblot analysis. However, mutation of Gly-203 abolished the ability to form multimers (Fig. 4A), supporting the conclusion that this residue is important in HifA–HifA interactions without affecting HifB–HifA interactions and HifA stability.

To extend these observations, we co-expressed the hifA Gly-203 mutants with pWW1 and then performed haemagglutination assays, whole cell dot immunoblot assays and transmission electron microscopy. As predicted from our analysis of multimer formation, HifAEag(G203A) and HifAEag(G203S) were not incorporated into mature pili (Fig. 4B). These results underscore the importance of the –14 glycine in assembly of H. influenzae pili.

The HifA subunit can be incorporated into functional pili by the heterologous pilus gene cluster from H. influenzae biogroup aegyptius strain F3031 and vice versa

To begin to identify other residues important in chaperone–subunit interactions or assembly of mature H. influenzae pili, we performed cross-complementation analysis of the pilus gene cluster from strain Eagan with the pilus assembly system in H. influenzae: biogroup aegyptius strain F3031 and vice versa. This approach would distinguish HifB Eag from HifB F3031 (Fig. 1A) by co-expressing pWW15ΔhifB and hifB-F3031 and then assessing pilus assembly. Interestingly, ORN103, harbouring both pWW15ΔhifB and pJS205 (hifB-F3031), was associated with haemagglutination titres and reactivity on dot immunoblots similar to those observed with co-expression of pWW15ΔhifB and the homologous chaperone (not shown).

Finally, we examined the role of residues that distinguish HifB Eag from HifB F3031 (Fig. 1A) by co-expressing pWW15ΔhifB and hifB-F3031 and then assessing pilus assembly. Interestingly, ORN103, harbouring both pWW15ΔhifB and pJS205 (hifB-F3031), was associated with haemagglutination titres and reactivity on dot immunoblots similar to those observed with co-expression of pWW15ΔhifB and the homologous chaperone (not shown).

Modelling of the HifA structure reveals marked sequence conservation in regions predicted to interact with HifB

To gain additional insight into the interactive surfaces of HifA and HifB, we began by aligning available HifA amino acid sequences and HifB homologues from a variety of bacterial species. A score matrix was used to align the HifA sequences from H. influenzae: biogroup aegyptius strain F3031 and other species. The HifA sequences were then subjected to a multiple sequence alignment program, and the alignment was manually edited to improve the accuracy of the results.

In addition, we examined the role of residues that distinguish HifB Eag from HifB F3031 (Fig. 1A) by co-expressing pWW15ΔhifB and hifB-F3031 and then assessing pilus assembly. Interestingly, ORN103, harbouring both pWW15ΔhifB and pJS205 (hifB-F3031), was associated with haemagglutination titres and reactivity on dot immunoblots similar to those observed with co-expression of pWW15ΔhifB and the homologous chaperone (not shown).

Fig. 5. A. Western analysis of periplasmic proteins from E. coli ORN103 containing either pWW1 (hifB-hifE_Eag) or pGK203 (hifB-hifE_F3031), complemented with either wild-type HifAEag (pJS206) or wild-type hifB-F3031 (pGK201), probed with anti-HifA antiserum. Samples were loaded as follows: lane 1, ORN103/pWW1 + pJS206; lane 2, ORN103/pWW1 + pGK201; lane 3, ORN103/pGK203 + pGK201; lane 4, ORN103/pGK203 + pJS206. The mature HifA species is indicated by an arrow.

B. Dot immunoblots of E. coli ORN103 with either pWW1 (hifB-hifE_Eag) or pGK203 (hifB-hifE_F3031), complemented with either wild-type HifAEag (pJS206) or hifB-F3031 (pGK201), probed with anti-HifB-HifA antiserum. Haemagglutination (HA) titres from the same ORN103 derivatives are indicated below the respective dot immunoblot samples.
acid sequences from 20 different \textit{H. influenzae} strains and then comparing these sequences with the amino acid sequence of the PapK subunit that was crystallized together with PapD (Sauer \textit{et al.},ting). As shown in Fig. 7A, using the strain Eagan HifA protein as a reference, alignment of the HifA sequences revealed striking homology between residues 1–61, 73–96, 103–132 and 167–197. As a corollary, maximal sequence diversity was concentrated in the three regions delimited by residues 62–72, 97–102 and 104–166. Interestingly, comparison of the HifA sequences and the PapK structure strongly suggests that HifA is a C-terminally truncated Ig fold. The two cysteines involved in disulphide bond formation, the hydrophobic core residues and all of the residues that participate in donor strand complementation in PapK are conserved between all of the HifA sequences and PapK.

Given the conservation of structural features between HifA and PapK, we chose to model the HifA structure based on the PapK crystal structure. Our goal was to address the level of amino acid sequence and structural conservation among HifA proteins in regions predicted to interact with the HifB chaperone. Figure 7B shows the hypothetical HifA structure. Yellow indicates structural residues that are conserved between HifA and PapK, whereas blue-purple indicates residues that are conserved among HifA sequences (with identity in at least 16 of the 20 sequences). The maximally divergent regions of HifA, including residues 62–72, 97–102 and 104–166, are coloured red and are shown as loops projecting away from the rest of the molecule. Of note, these regions represent insertions relative to the PapK sequence, thus precluding accurate modelling of their conformation.

Examination of the hypothetical HifA structure together with a chaperone reveals high level sequence and structural conservation in all areas potentially interacting with the chaperone. In contrast, the hypervariable regions are confined to three separate sites that are unlikely to interact with the chaperone. These regions are exposed on the surface of the molecule and are potentially exposed in assembled pili. Of note, previous investigators identified three relatively hydrophilic regions within HifA sequences and originally proposed that these regions might constitute highly conserved antigenic epitopes (Forney \textit{et al.}, 1991). However, in subsequent studies, antiseras raised against peptides corresponding to these regions failed to react with native pili (Gilsdorf \textit{et al.}, 1993). Based on the hypothetical HifA structure, these three regions (corresponding to amino acids 15–33, 79–95 and 122–132 in the mature Eagan HifA protein) contribute to the A, C and D \(\beta\)-sheets and are likely to be obscured in mature pili. In additional work, Palmer and Munson described a monoclonal antibody designated 3H12 that appeared to react specifically with an epitope present in strain M37 pili and absent from strain MinnA pili (1992). Mapping of this strain-specific epitope localized it to a region that includes the two hypervariable regions between amino acids 62–72 and 97–102 (Palmer and Munson, 1992), providing strong evidence that these regions are surface-exposed in native pili.

\textbf{Discussion}

\textit{H. influenzae} haemagglutinating pili possess a number of characteristics reminiscent of P pili. In particular, they are composite fibres consisting of a thick rod joined to a thin tip fibrillum; their assembly involves a periplasmic chaperone.
(HifB) and an outer membrane usher (HifC); and purified complexes containing HifB and the major structural subunit (HifA) spawn the spontaneous self-assembly of HifA into rod-like structures (St. Geme et al. 1996; Gilsdorf et al., 1997). On the other hand, two significant departures from the P pilus system include a two-stranded architecture and a greater economy of genes necessary for pilus assembly (5 rather than 11) (van Ham et al., 1994; St. Geme et al., 1996). Whether the molecular basis of chaperone–subunit and subunit–subunit interactions might be different has been unclear.

More than 30 PapD-like periplasmic chaperones have been identified, including the HifB chaperone involved in the assembly of H. influenzae haemagglutinating pili. In the present study, we targeted the HifB Arg-9 and Lys-119 residues, which are invariant among members of the PapD superfamily and, in the case of PapD, participate in donor strand complementation (Hung et al., 1996; Sauer et al., 1999). Of note, mutation of these residues abolished HifB–HifA interactions and eliminated formation of pili. In P pilus subunits, the penultimate tyrosine residue interacts with the G1 strand of PapD and is critical for pilus assembly. Comparison of HifA sequences from 20 different strains reveals a penultimate tyrosine in all cases. Interestingly, when this amino acid was replaced by another aromatic residue in the Eagan HifA protein, productive interaction between HifA and HifB was still possible and pilus assembly proceeded, although with...
slightly reduced efficiency. In contrast, substitution with serine abolished pili formation. Thus, substitutions at canonical positions in either the chaperone or the subunit disrupt HifB–HifA complexes, arguing that HifB stabilizes subunits via a donor strand complementation mechanism.

Subunits assembled by the chaperone/usher pathway possess an N-terminal extension (residues 1–13 in PapK) with a highly conserved motif that has been shown to participate in subunit–subunit interactions (Soto et al., 1998). This motif consists of alternating hydrophobic amino acids and is similar to the PapD G1 strand involved in donor strand complementation. Based on the crystal structure of PapK, the N-terminal extension does not contribute to the Ig fold, instead projecting away from the fold and free to interact with another subunit (Sauer et al., 1999). With this information in mind, during pilus biogenesis, the N-terminal extension of one subunit is believed to displace the chaperone G1 strand from the neighbouring subunit in a process termed donor strand exchange (Choudhury et al., 1999; Sauer et al., 1999). Accordingly, the mature pilus should consist of an array of perfectly canonical Ig folds, each containing a strand from the preceding subunit. In this context, it is noteworthy that mutation of the PapA –14 residue from glycine to alanine changed the helical symmetry of pili, and mutation from glycine to tyrosine reduced assembly of pili (Bullitt et al., 1996). Similarly, mutation of the HifA –14 glycine to either alanine or serine completely disrupted HifA polymerization, suggesting that assembly of H. influenzae haemagglutinating pili may also occur via donor strand exchange.

Comparison of HifA amino acid sequences from multiple strains demonstrates significant diversity. Despite this diversity, in cross-complementation studies, we found that the HifB chaperone from strain Eagan was capable of interacting with both the strain Eagan HifA and the strain F3031 HifA. Similarly, the HifB chaperone from strain F3031 demonstrated productive interaction with both the strain F3031 and the strain Eagan HifA molecules. Considered together, these observations suggest that residues involved in donor strand complementation and donor strand exchange are conserved among different Hif subunits and HifB chaperones. Consistent with this possibility, in the modelled HifA structure, regions predicted to participate in subunit–subunit interactions (Soto et al., 1999) argue that at least two of the three hypervariable regions are exposed on native pili. In particular, this antibody reacts specifically with an epitope present in native pili from H. influenzae strain M37 but not other strains, and mapping of this epitope has localized it to a region that includes the two hypervariable regions between amino acids 62–72 and 97–102 (Palmer and Munson, 1992).

There are several potential limitations to studying H. influenzae pili expression in E. coli rather than the parental background. Considerations include the decoupling of bidirectional transcription by co-expressing hifA and hifB on different plasmids, over-expression of the major structural subunit (HifAEag or HifA<sub>F3031</sub>), resulting in saturation of chaperones and usher sites and interference with the efficient export of HiD or HiE, and the loss of possible regulatory units and feedback mechanisms that may modulate pilus assembly. In light of these limitations, we restricted our goals in this study to dissecting the structural details of pilus biogenesis by site-directed mutagenesis and reconstitution of pilus assembly. Further details related to pilus assembly and function may necessitate reconstitution in the original H. influenzae background.

To summarize, our results emphasize the broad applicability of the paradigm for pilus biogenesis identified in studies of P pili, including the concepts of donor strand complementation and donor strand exchange. In addition, they provide new information about the structure of the HifA subunit and suggest a structural explanation for antigenic variation in H. influenzae haemagglutinating pili.

**Experimental procedures**

**Bacterial strains and plasmids**

H. influenzae strain Eagan is a type b strain that contains the intact hif gene cluster (hif<sub>Eag</sub>) (McCrea et al., 1994). H. influenzae biogroup aegyptius strain F3031 contains the hif<sub>F3031</sub> gene cluster, which is homologous to the hif<sub>Eag</sub> gene cluster (Read et al., 1996). E. coli DH5<sub>x</sub> was used for cloning purposes (Sambrook et al., 1989) and ORN103 served as the host strain for all complementation studies (Orndorf and Falkow, 1984). The plasmid constructs used in this study are described in Table 1. Plasmids pJS201 and pJS206 contain the hif<sub>Eag</sub> gene as an EcoRI–BamHI fragment and are identical except for the vector backbone and the corresponding inducible promoter. Similarly, pJS202 and pJS204 contain the hif<sub>Eag</sub> gene as an EcoRI–BamHI fragment and...
are identical except for the vector backbone and the inducible promoter. To construct pJS205, the hifB3031 gene was amplified from strain F3031 chromosomal DNA using the polymerase chain reaction (PCR) and then cloned into the polylinker site downstream of the tac promoter in pMMB91. To construct pGK201 and pGK202, the hifA3031 gene was liberated from pAW513 as an EcoRI HindIII fragment and cloned into the polylinker downstream of either the tac promoter in pTrc99A or the tac promoter in pMMB91. For studies of the in vitro assembly of strain Eagan pili, pWW15 and pWW1 were employed. pWW15 contains the strain Eagan pilus gene cluster cloned into the vector pOK12. pGK203 is a derivative of pWW15 truncated at the hifA BglII site (McCrea et al., 1994). For studies of the in vitro assembly of strain F3031 pili, pMF20 and pGK202 were used. pMF20 contains a 14 kb BglII fragment with the entire strain F3031 pilus gene cluster cloned into the BglII site of the vector pOK12. pGK202 contains the 5' end of hifA3031 together with hifB-hifE3031 and was constructed by digesting pMF20 with PvuII and BglII and then cloning the fragment into PvuII–BglII-digested pGEM5.

**Table 1. Plasmid constructs.**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM5</td>
<td>AmpR</td>
<td>Promega</td>
</tr>
<tr>
<td>pMMB91</td>
<td>KanR</td>
<td>Viera and Messing (1991)</td>
</tr>
<tr>
<td>pOK12</td>
<td>KanR</td>
<td>Amann et al. (1988)</td>
</tr>
<tr>
<td>pTrc99A</td>
<td>AmpR</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUC19</td>
<td></td>
<td>Whitney and Farley (1993)</td>
</tr>
<tr>
<td>pWW15</td>
<td>pGEM5-hifA</td>
<td>St. Gme et al. (1996)</td>
</tr>
<tr>
<td>pJS201</td>
<td>pTrc99A-hifA (Y215F)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS201.1</td>
<td>pTrc99A-hifA (Y215S)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS201.2</td>
<td>pTrc99A-hifA (Y215Stop)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS201.3</td>
<td>pTrc99A-hifA (G203A)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS201.4</td>
<td>pTrc99A-hifA (G203S)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS201.5</td>
<td>pTrc99A-hifA (G203S)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS202</td>
<td>pMMB91-hifB</td>
<td>St. Gme et al. (1996)</td>
</tr>
<tr>
<td>pJS202.1</td>
<td>pMMB91-hifB (K119A)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS202.2</td>
<td>pMMB91-hifB (R9G)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS204</td>
<td>pTrc99A-hifB</td>
<td>This study</td>
</tr>
<tr>
<td>pJS205</td>
<td>pMMB91-hifB (K119A)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS206</td>
<td>pMMB91-hifB</td>
<td>This study</td>
</tr>
<tr>
<td>pJS206.1</td>
<td>pMMB91-hifB (Y215F)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS206.2</td>
<td>pMMB91-hifB (Y215S)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS206.3</td>
<td>pMMB91-hifB (Y215Stop)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS206.4</td>
<td>pMMB91-hifB (G203A)</td>
<td>This study</td>
</tr>
<tr>
<td>pJS206.5</td>
<td>pMMB91-hifB (G203S)</td>
<td>This study</td>
</tr>
<tr>
<td>pGK201</td>
<td>pMMB91-hifA</td>
<td>This study</td>
</tr>
<tr>
<td>pGK202</td>
<td>pTrc99A-hifB (F3031)</td>
<td>This study</td>
</tr>
<tr>
<td>pMF20</td>
<td>pOK12-hifA-hifE (E3031)</td>
<td>This study</td>
</tr>
<tr>
<td>pGK203</td>
<td>pGEM5-hifA-PvuI-hifE (E3031)</td>
<td>This study</td>
</tr>
<tr>
<td>pWW15</td>
<td>pGEM5-hifA-hifE (E3031)</td>
<td>McCrea et al. (1994)</td>
</tr>
<tr>
<td>pWW15ΔhifB</td>
<td>pWW15 with in frame hifB deletion</td>
<td>This study</td>
</tr>
<tr>
<td>pWWW1</td>
<td>pGEM5-hifA-RsrI-hifE (E3031)</td>
<td>Watson et al. (1994)</td>
</tr>
</tbody>
</table>

Initially, two separate reactions were performed using pJS201 as the template, including one with the 5' primer and one mutagenic primer and the second with the 3' primer and the complementary mutagenic primer. The resulting fragments were then combined as the template for a reaction performed with the 5' and 3' primers. hifAseq mutations were generated more directly by introducing the mutation of interest into the hifAseq 3' primer and then amplifying the entire gene.

To create an in frame deletion in hifBseq in the context of the rest of the hifAseq gene cluster, we modified the plasmid pWW15. Recombinant PCR was used to generate a BglII–EcoRI fragment, corresponding to the 5' end of hifAseq, the hifBseq gene with an internal 465-base pair deletion and the 5' end of hifCseq. Subsequently, this fragment was inserted in place of the 2.94 kb BglII–EcoRI fragment in pWW15, generating pWW15ΔhifB.

**Haemagglutination assays and pilus enrichment**

*E. coli* transformants were incubated in Luria–Bertani broth at 37°C to an A600nm of 0.8, and gene expression (under control of the tac or trc promoter) was induced with 1 mM isopropylthio-β-d-galactoside (IPTG) for 1 h. To select for piliated organisms, enrichment was performed using a suspension of human erythrocytes, as has been previously described (Connor and Loeb, 1983). Criteria for pili expression included a haemagglutination titre ≥1:8 (Pichichero et al., 1982), the presence of pili by negative staining transmission electron microscopy, reactivity on whole cell dot blots, and 1:8 (Pichi-

© 2000 Blackwell Science Ltd, Molecular Microbiology, 35, 1335–1347
immunoblots and inhibition of haemagglutination by pre-
incubation with 100 μg ml⁻¹ of GM1 (Sigma), a sialylated
ganglioside that appears to mimic the pilus receptor structure
(van Alphen et al., 1991).

Negative staining transmission electron microscopy

Suspensions of bacteria were collected from induced cultures
and negative staining was performed with 0.5% uranyl acetate,
as has been previously described (St. Geme et al., 1991).
Samples were examined using a Zeiss 10A transmission
electron microscope.

Cell fractionation and protein analysis

Periplasmic extracts were prepared as has been previously
described (Slonim et al., 1992) and resolved on 12% polyacrylamide
gels using SDS–PAGE. Samples were then
electro-transferred to nitrocellulose membranes, which were
blocked with 5% skim milk in Tris-buffered saline (TBS) and
then probed with a rabbit polyclonal antiserum raised against
either purified HiIB obtained from strain Eagan or denatured
HiA recovered from strain Eagan (St. Geme et al., 1996).
Goat antirabbit IgG conjugated to alkaline phosphatase was
employed as the secondary antibody, and reactivity was
detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-
indolyl phosphate (Kirkegaard and Perry Laboratories).

In performing whole cell immunoblot assays, organisms
were first fixed for 30 min with 4% paraformaldehyde in
phosphate buffered saline (PBS). These organisms were
subsequently washed three times with PBS, and suspensions
were adjusted to an A₆₀₀nm of 0.6. Next, 100 μl volumes were applied to a nitrocellulose membrane resting
on a single blotting sponge (VWR) in a Manifold I micro-

cell filtration manifold apparatus (Schleicher and Schuell).
After incubation for 30 min, low suction was applied to draw
residual buffer through the manifold. The membrane was
blocked in 5% skim milk in TBS for one hour, then probed
with a rabbit polyclonal antiserum raised against the purified
HiIB–HiA complex from strain Eagan (St. Geme et al., 1996),
an antiserum that is cross-reactive with strain F3031 pil.
Goat antirabbit IgG conjugated to horseradish peroxidase
(Sigma) was used as the secondary antibody and reactivity
was detected using chemiluminescence with the SuperSignal
enhanced substrate (Pierce) together with autoradiography
film. As a control for membrane integrity, we performed
simultaneous whole cell immunoblots with the anti-HiIB
antiserum, which should not react with whole cells as HiIB
is confined to the periplasm.

HiA modelling

Mutational analysis and sequence alignments indicated that
HiA probably adopts a truncated Ig fold similar to that seen in
the PapK structure. With this understanding, we used the
PapK structure in the PapD–PapK complex (PDB accession
code 1PDK) to model the structure of HiA. The PDB coordi-
nates were not altered. Residues in PapK were colour coded
according to sequence conservation, as described in the
legend of Fig. 7, to reveal the likely locations of both
conserved and variable regions in HiA.

Acknowledgements

This work was supported by Public Health Service grants 1RO1
DC-02873 and 1RO1 AI-44167 (J.W.S.). G.P.K. was supported
by a fellowship from the Pediatric Infectious Diseases Society
and by a Young Investigator’s Matching Grant from the National
Foundation for Infectious Diseases.

References

van Alphen, L., Poole, J., and Overbeeke, M. (1986) The Anton
blood group antigen is the erythrocyte receptor for Haemo-

van Alphen, L., Geelen-van den Broek, L., Blaas, L., van Ham,
adherence of Haemophilus influenzae by sialyl gangliosides.

Amann, E., Ochs, B., and Abel, K.J. (1988) Tightly regulated tac
promoter vectors useful for the expression of unfused and

Brinton, C.C. Jr, Carter, M.J., Derber, D.B., Kar, S., Kramarik,
vaccines for Haemophilus influenzae diseases. Pediatr Infect
Dis J 8: 554–561.

Bullitt, E., Jones, C.H., Striker, R., Soto, G., Jacob-Dubuisson, F.,
subassemblies in vitro depends on chaperone uncapping of a


Choudhury, D., Thompson, A., Stojanoff, V., Langermann, S.,
structure of the FimC-FimH chaperone-adhesin complex from

Clemans, D.L., Marrs, C.F., Patel, M., Duncan, M., and Gilsdorf,

for detection of colonies of Haemophilus influenzae type b

Farley, M., Stephens, D.S., Kaplan, S.L., and Mason, Jr, E.O.
(1990) Pilus- and non-pilus–mediated interactions of Haemo-
philus influenzae type b with human erythrocytes and human

Comparison and analysis of the nucleotide sequences of pilin
genes from Haemophilus influenzae type b strains Eagan and

Furste, J.P., Pansegrau, W., Frank, R., Bloker, H., Scholz, P.,
for type b strains of Haemophilus influenzae type b and human

and nonconserved epitopes among Haemophilus influenzae

Gilsdorf, J.R., Chang, H.Y., McCrea, K.W., and Bakalcz, L.O.
(1992) Comparison of hemagglutinating pilus of Haemophilus
influenzae type b with similar structures of nontypeable H.

of antibodies against conserved regions of pilins of Haem-


