

Chaperone-Subunit-Usher Interactions Required for Donor Strand Exchange during Bacterial Pilus Assembly

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The assembly of type 1 pili on the surface of uropathogenic *Escherichia coli* proceeds via the chaperone-usher pathway. Chaperone-subunit complexes interact with one another via a process termed donor strand complementation whereby the G1 β strand of the chaperone completes the immunoglobulin (Ig) fold of the pilus subunit. Chaperone-subunit complexes are targeted to the usher, which forms a channel across the outer membrane through which pilus subunits are translocated and assembled into pili via a mechanism known as donor strand exchange. This is a mechanism whereby chaperone uncapping from a subunit is coupled with the simultaneous assembly of the subunit into the pilus fiber. Thus, in the pilus fiber, the N-terminal extension of every subunit completes the Ig fold of its neighboring subunit by occupying the same site previously occupied by the chaperone. Here, we investigated details of the donor strand exchange assembly mechanism. We discovered that the information necessary for targeting the FimC-FimH complex to the usher resides mainly in the FimH protein. This interaction is an initiating event in pilus biogenesis. We discovered that the ability of an incoming subunit (in a chaperone-subunit complex) to participate in donor strand exchange with the growing pilus depended on a previously unrecognized function of the chaperone. Furthermore, the donor strand exchange assembly mechanism between subunits was found to be necessary for subunit translocation across the outer membrane usher.

Type 1 pili are assembled via the highly conserved chaperone-usher pathway, which directs the synthesis of over 30 different adhesive organelles expressed by a multitude of pathogenic bacteria (20, 45). Two prototypical members of the chaperone-usher pathway are type 1 and P pili, which are produced by uropathogenic *Escherichia coli*. Both of these adhesive organelles are important in mediating attachment to epithelial cells during urinary tract infections. Type 1 pili bind to mannose residues present on the bladder epithelium via the FimH adhesin (18) and are therefore critical for the establishment of cystitis (9, 25, 28, 33, 51). P pili mediate binding to Gal α (1-4)Gal disaccharides present on the kidney epithelium via the PapG adhesin and are important for the development of pyelonephritis (5, 11, 26, 29, 36, 47, 52). Genes important for type 1 pilus production are encoded by the *fim* gene cluster (*fimA* to *fimH*) (35, 49), while genes important for P pili are encoded by the *pap* gene cluster (*papA* to *papG*) (15, 37). Both of these pili are heteropolymeric structures composed of rigid pilus rods joined to thinner and more flexible tip fibrillae with the tip fibrillum of type 1 pili being short and stubby in comparison to that of P pili (22, 26). The type 1 pilus rod is comprised of repeating monomers of FimA arranged in a right-handed helical cylinder, and the tip fibrillum is composed of FimF, FimG, and the adhesin FimH (6, 22).

Both the chaperone and the usher, FimC and FimD in the type 1 pilus system, are absolutely required for pilus biogenesis to occur, as mutations in both genes result in nonpiliated bacteria (24, 30). Mutations in *fimC* result in the degradation

of pilus subunits in the periplasm (21, 30), and mutations in *fimD* result in the accumulation of chaperone-subunit complexes in the periplasm (24, 34). The chaperone interacts with pilus subunits in a mechanism called donor strand complementation, which facilitates subunit folding on the chaperone template (8, 38). Pilus subunits are incomplete immunoglobulin (Ig) folds, resulting in a groove or scar present in the subunit exposing its hydrophobic core. In complex with the chaperone, the Ig fold of the pilus subunit is completed by the G1 β strand of the chaperone, which is inserted into the groove and protects the hydrophobic core of the subunit. The chaperone completes the Ig fold of the subunit in an atypical fashion by inserting its G1 β strand parallel to the F strand of the subunit. The folded subunit remains bound to the chaperone so that the folding reaction is simultaneously coupled with the capping of the interactive groove of the subunit (7, 27, 44), stabilizing the subunit in an assembly-competent conformation.

During type 1 pilus assembly, chaperone-subunit complexes are targeted to the usher FimD, which forms a pore in the outer membrane allowing the translocation of pilus subunits to the cell surface (24, 42, 50). Pilus subunits are targeted to the usher in a specific order reflecting their final order in the pilus (10, 42). The chaperone-adhesin complex binds first, most tightly, and fastest to the usher, suggesting that adhesins initiate pilus assembly (10, 41, 42). In support of this hypothesis, clinical isolates lacking a functional *fimH* gene did not produce type 1 pili (9, 28). Furthermore, the binding of FimC-FimH to FimD induces a conformational change in FimD, presumably into an assembly-competent state (42). At the usher, pilus assembly is thought to occur by a reaction termed donor strand exchange (3, 8, 38). Donor strand exchange predicts that the N-terminal extension of one subunit will displace the G1 β strand of the chaperone from its neighboring subunit and in-

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sert itself antiparallel to the F strand of the subunit to complete a canonical Ig fold. Thus, the pilus would consist of perfectly canonical Ig domains, each of which contributes a strand to the fold of its neighboring subunit. In support of the donor strand exchange hypothesis, fusion of the N-terminal extension of FimG onto the C terminus of FimH created donor strand-complemented FimH (dscFimH), which was stable in the periplasm in the absence of FimC and folded on its own (3). In contrast, FimH required FimC for proper folding and stability in the periplasm (3). In addition, we have recently cocrystallized and solved the structure of the N-terminal extension of PapK bound to the PapE pilin (40). The structure revealed that the N-terminal extension completes the Ig fold of the pilin in a canonical fashion, thus proving the donor strand exchange hypothesis. Thus, pilus subunits do not contain all of the information necessary for folding, and this missing information is supplied by the chaperone during donor strand complementation and by the N-terminal extension of neighboring subunits in the pilus fiber. The chaperone primes the subunit for assembly by holding the groove in an open, activated conformation. During donor strand exchange, the subunit undergoes a topological transition that triggers the closure of the groove and seals the N-terminal extension in place. It is this topological transition, made possible only by the priming action of the chaperone, which drives subunit assembly into the fiber (40).

Here, we elucidated factors governing donor strand exchange, assembly, and translocation across the outer membrane usher. We also demonstrated that the information required for targeting the FimC-FimH complex to the usher was contained primarily within the FimH protein and did not require the FimC chaperone. FimH is the first protein to be incorporated into the pilus, and its interaction with the usher initiates pilus assembly (10, 41, 42). The incorporation of subsequent subunits into the pilus and their translocation across the usher depend on a previously unrecognized function of the chaperone necessary to facilitate the donor strand exchange process.

MATERIALS AND METHODS

Genetic constructs. To create pTrc-FimGH, FimG was PCR amplified from pETS2A (42) by using the two oligonucleotides FimGH top (5'-CCATCGATTATTGGCGTGACTTTTGTATCAAGATAACAAACAGGATGCACCA TCACG-3') and FimG bot (5'-CGCGGATCCTCAGCTGTAGGTATAGGTG ATGCT-3'). This PCR product was cloned into the *Cla*I and *Bam*HI sites of pTrc-dscFimH (3), creating pTrc-FimGH. FimGH was subcloned from pTrc-FimGH into pMMB91 by using the *Eco*RI and *Bam*HI sites to create pMMB-FimGH. FimGH was also subcloned from pTrc-FimGH into pBad18-Kn (13) by using the *Eco*RI and *Xba*I sites to create pBad-FimGH. pMB10 was constructed by subcloning FimGH from pBad-FimGH with the *Sph*I and *Nhe*I sites into the *Sph*I and *Xba*I sites of pBad33 (13). FimG was PCR amplified from pETS2A by using the two oligonucleotides FimG top (5'-CCGGAATTCATGAAATGGTG CAAACGTGGG-3') and FimG bot (above), and dscFimG was PCR amplified by using the two oligonucleotides FimG top (above) and dscFimG bot (5'-CG CGGATCCTTATGTTATCCCTGACATAGCCGCGGATAGTAATCGTGCT ATCACTTGT-3'). The PCR products were cloned into the *Eco*RI and *Bam*HI sites of pTrc99A (2), creating pTrc-FimG and pTrc-dscFimG, respectively. FimF was PCR amplified from pETS9 (41) by using the two oligonucleotides FimF top (5'-GGATCCAAGGGGCGGTGATGAGAAACA-3') and FimF bot (5'-CTGCAGTTACTGATATCAAGAGTGAA-3'). The FimF PCR product was cloned into the *Bam*HI and *Pst*I sites of pTrc99A (2), creating pTrc-FimF. The FimH receptor binding domain was PCR amplified from pBad-FimH (3) by using the two oligonucleotides FimH top *Eco*RI (5'-CCCGAATT CACCCGAGGGGATGATTGTAAT-3') and T2HA bot (5'-TCTAGATTAAG

TABLE 1. List of plasmids used in this work

Plasmid	Gene(s)	Reference
pMMB66	Cloning vector	12
pMMB-FimH	<i>fimH</i>	18
pMMB-dscFimH	<i>dscFimH</i>	This report
pMMB-FimGH	<i>fimGH</i>	This report
pCD	<i>fimCfimD_{his}</i>	Saulino and Hultgren, unpublished
pCD-Kn	<i>fimCfimD_{his}</i>	This report
pETS1007	<i>fimC fimH</i>	41
pMB7	<i>fimH</i>	This report
pMB10	<i>fimGH</i>	This report
pBad18-Kn	Cloning vector	13
pBad-FimH	<i>fimH</i>	3
p421	<i>fimH_p</i>	This report
p601	<i>fimH_r</i>	This report
pBad-FimGH	<i>fimGH</i>	This report
pTrc99A	Cloning vector	2
pTrc-FimH	<i>fimH</i>	3
pTrc-FimG	<i>fimG</i>	This report
pTrc-dscFimG	<i>dscFimG</i>	This report
pTrc-FimF	<i>fimF</i>	This report
pTrc-FimGH	<i>fimGH</i>	This report
pUT2002	<i>fimH</i> mutant type 1 gene cluster	32
pETS7	<i>fimD_{his}</i>	41
pETS1000	<i>fimC_{his}</i>	42
pHJ9205	<i>fimC</i>	C. H. Jones and S. J. Hultgren, unpublished
pMC10	<i>csgE-HA</i>	M. Chapman and S. J. Hultgren, unpublished

CGTAGTCCGGAACGTCGTACGGGTAGCCGCCAGTAGGCACCACCA CATCATTATTGGC-3'). The PCR product was cloned into the *Eco*RI and *Xba*I sites of pBad18-Kn to create p601, which expresses the FimH receptor binding domain with a hemagglutinin (HA) tag at its C terminus. An HA tag was put on the FimH pilin domain by annealing together the two oligonucleotides HA top (5'-CTACCCGTACGACGTTCCGGACTACGCTGTAC-3') and HA bot (5'-AGCGTAGTCCGGAACGTCAGTACGGGTAGGTAC-3') and ligating them into the *Kpn*I site of pUC-ssCOOHFimH (D. L. Hung and S. J. Hultgren, unpublished data). This plasmid was then digested with *Eco*RI and *Xba*I and ligated into the same sites in pBad18-Kn, creating p421, which expresses the FimH pilin domain with an HA tag at its N terminus. dscFimH was subcloned from pUC-dscFimH (3) into pMMB66 by using the *Eco*RI and *Bam*HI sites to create pMMB-dscFimH. FimH was subcloned from pBad-FimH (3) with the *Nhe*I and *Hind*III sites into the *Xba*I and *Hind*III sites of pBad33 (13), creating pMB7. FimC and FimD_{His} were subcloned from pCD (E. T. Saulino and S. J. Hultgren, unpublished data) with the *Apa*I and *Pst*I sites into pMMB91 to create pCD-Kn. All constructs made by PCR were sequenced. The plasmids used in this study are listed in Table 1, and a schematic of the gene products is shown in Fig. 1.

Periplasmic preparations. Bacterial strains were grown to an optical density at 600 nm of 0.8 and induced with 0.1% arabinose and 0.05 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 1 h. Periplasms were prepared as described previously (43), and samples were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by Western blotting with anti-FimCH antiserum (MedImmune, Inc.). FimC-FimH antibodies are polyclonal and were generated against pure FimC-FimH complexes.

In vitro subunit-subunit binding assays. FimC_{His}-FimG complexes were purified from the strain C600/pETS1000/pETS2A as described previously (42). dscFimG was purified from the periplasm of strain C600/pTrc-dscFimG by using anion exchange and hydrophobic interaction columns. Pure FimH was obtained as described previously (3). FimH in 3 M urea was diluted 10-fold in the presence of dscFimG or FimC_{His}-FimG complexes followed by incubation with excess FimH. dscFimG-FimH or FimC_{His}-FimG-FimH complexes were further purified by cation-exchange chromatography. dscFimG-FimH or FimC_{His}-FimG-FimH complexes were run on an SDS-polyacrylamide gel stained with Coomassie blue after incubation in SDS at either 95 or 25°C. dscFimG-FimH or FimG-FimH complexes can be detected because they must be incubated at 95°C to dissociate (46).

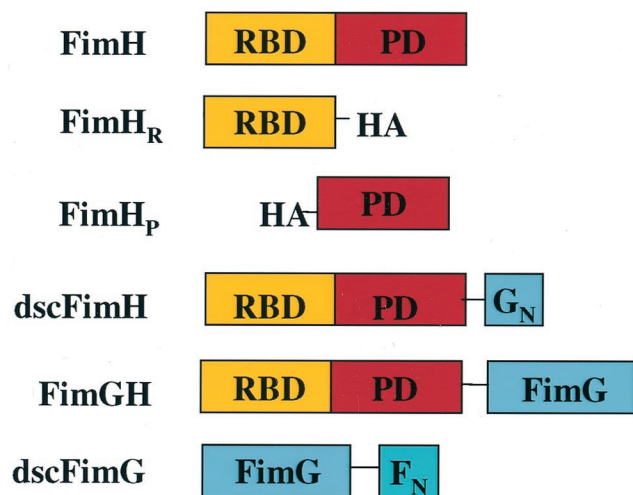


FIG. 1. Schematic diagram of some of the constructs used in this work. FimH_R is the FimH receptor binding domain with an HA tag at its C terminus. FimH_P is the FimH pilin domain with an HA tag at its N terminus. dscFimH has the first 13 amino acids of FimG fused onto its C terminus with a 4-amino-acid linker sequence, DNKQ, preceding the FimG sequence (FimG amino terminus, DVTITVNGKVVAK) (3). FimGH was constructed identically to dscFimH, except that it contains the entire FimG sequence instead of just the first 13 amino acids of FimG. dscFimG has the first 13 amino acids of FimF fused onto its C terminus with a 4-amino-acid linker sequence, DNKQ, preceding the FimF sequence (FimF amino terminus, DVSTITIRG YVRDN). Abbreviations: RBD, receptor binding domain; PD, pilin domain; G_N, FimG N-terminal extension; F_N, FimF N-terminal extension.

Outer membrane preparation and usher copurification. Usher copurification experiments were performed by expressing FimC and the appropriate subunit(s) with FimD_{His} (41, 42). Bacterial strains were grown to an optical density at 600 nm of 0.8 and induced with 0.1 mM IPTG and 0.1% arabinose for 1 h. The cells were harvested, and outer membranes were prepared as described previously (48, 50). Nickel-nitrilotriacetic acid beads (Qiagen) were added to the outer membranes and rocked overnight at 4°C. The beads were washed several times in buffer containing 0.3 M NaCl, 20 mM HEPES (pH 7.4), and 0.1% dodecyl maltoside with 10 to 20 mM imidazole. Protein was eluted from the beads by using the same buffer containing 30 to 100 mM EDTA instead of imidazole. Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie blue or Western blotting with anti-FimCH (MedImmune, Inc.), anti-FimD (MedImmune, Inc.), anti-FimF (Soman Abraham), anti-FimG (1), and/or anti-HA (Convance) antiserum. FimD antibodies are polyclonal and were generated against native protein. FimF and FimG antibodies were generated against peptides.

Hemagglutination assays. The strain Orn103/pUT2002 (32) was transformed with pTrc99A, pTrc-FimH, or pTrc-FimGH and grown statically in the presence of 0.1 mM IPTG for 48 h. The cells were harvested, and hemagglutination assays were performed as described previously (16). Results are representative of three independent experiments.

RESULTS

FimH has sufficient information for FimD binding. The ability of dsc pilus subunits to bind to the usher was measured to test the requirement of the chaperone in usher binding. dsc subunits have the amino-terminal extension of their respective neighboring subunit fused to their C terminus (Fig. 1). The presence of this added β strand completes the Ig fold of the subunit and alleviates the requirement of the chaperone for folding and stability in the periplasm (3). The FimH adhesin is the first subunit incorporated into the pilus, and FimC-FimH

complexes bind first, most tightly, and fastest to FimD (42). The PapG adhesin of the P pilus system also binds first to its cognate usher protein (10). Since pilus adhesins bind first to the usher, we hypothesized that the primary information necessary for usher binding resided in the adhesin protein itself. To test this hypothesis, the ability of dscFimH (Fig. 1) to bind to FimD was determined. dscFimH, FimH, or FimC-FimH was coexpressed with FimD_{His}, and outer membrane extracts were subjected to nickel chromatography. FimD_{His} expressed by itself and purified by nickel chromatography is shown in Fig. 2A, lane 1. The lower-molecular-weight bands represent FimD degradation products. FimH expressed in the absence of FimC did not copurify with FimD (Fig. 2A, lane 2) because FimH is unstable in the absence of FimC. dscFimH copurified with FimD (Fig. 2A, lane 4), as did FimC-FimH complexes (42) (Fig. 2A, lane 3). These results suggest that FimC is not required for FimH binding to FimD and that folded FimH has sufficient information necessary for FimD binding.

Pilus adhesins are distinct from other pilus subunits because they contain an extra domain, an N-terminal receptor binding domain that is linked to the pilin domain. We hypothesized that the FimH receptor binding domain is the portion of FimH that allows it to bind to FimD first because the pilin domain must remain accessible, and exposed in the periplasm, in order to participate in donor strand exchange with the subsequent incoming subunit (FimG). The receptor binding (FimH_R) and pilin (FimH_P) domains of FimH were individually cloned (Fig. 1), and the ability of these two proteins to bind to FimD was assessed. Both FimH_R and FimH_P were tagged with HA for detection by Western blotting. FimH_R was stable in the periplasm, and FimH_P required FimC for stability in the periplasm as expected (data not shown). Both FimH_R and FimH_P copurified with FimD_{His} (Fig. 2B and D, lanes 3 and 4) as did FimC-FimH complexes (Fig. 2B and C, lanes 2). Surprisingly, no detectable FimC copurified with FimH_P-FimD complexes (Fig. 2C and D, lanes 4). FimC stabilizes subunits via donor strand complementation and accordingly is required for FimH_P stability. It is unclear why or when FimC dissociates during the purification process. Nevertheless, FimH_P clearly binds to FimD, indicating that FimH_P has information necessary for FimD binding. As a control an unrelated HA-tagged periplasmic protein, CsgE, was coexpressed with FimD_{His}. Although CsgE is stably expressed in the periplasm, it did not copurify with FimD_{His} (Fig. 2B and D, lanes 5). These results demonstrate that both the FimH_R and FimH_P domains contain surfaces that bind to FimD. Since FimH is L shaped (8), we propose that FimH_R is inserted into the usher pore while the FimH_P domain would then straddle the periplasmic surface of FimD (Fig. 5). Presumably, the groove occupied by the chaperone is oriented toward the periplasm in order to facilitate a donor strand exchange reaction with an incoming FimC-FimG complex.

The chaperone is required for donor strand exchange. Next, we wanted to analyze the ability of a dsc subunit to be incorporated into a growing pilus to determine if the chaperone is required during the donor strand exchange reaction. dscFimH was not used in these studies because after it binds to the usher pilus assembly is terminated, since its interactive groove is already filled with the added donor strand (3). Thus, dscFimG was constructed to test the role of the chaperone during donor

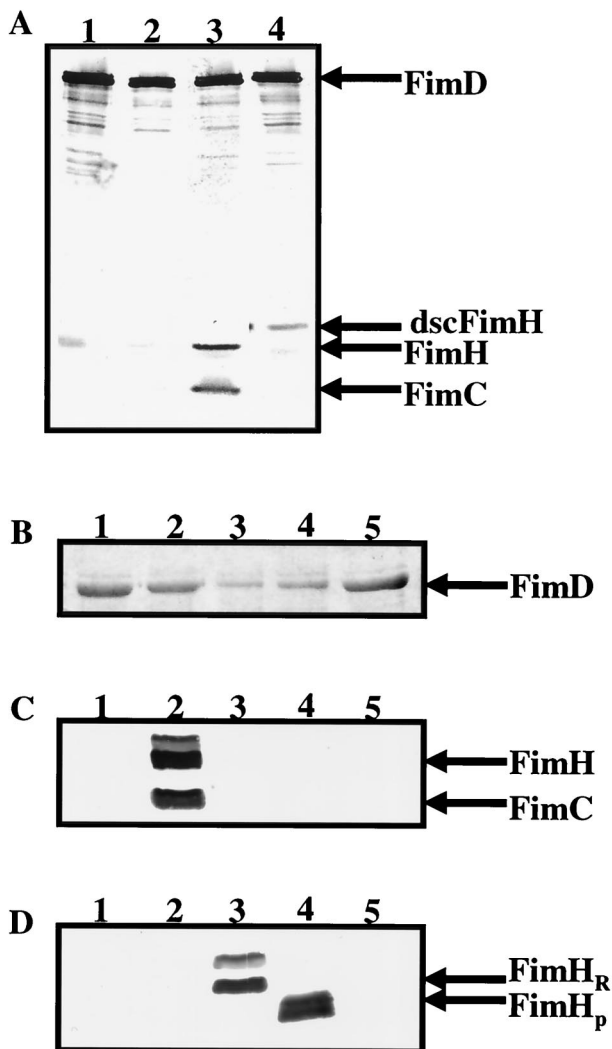


FIG. 2. FimH has sufficient information for FimD binding. (A) Western blot developed with anti-FimCH and anti-FimD antiserum of outer membrane preparations after nickel chromatography from the strains C600/pETS7/pMMB66 (FimD_{His}) (lane 1), C600/pETS7/pMMB-FimH (FimD_{His} + FimH) (lane 2), C600/pETS7/pETS1007 (FimD_{His} + FimC + FimH) (lane 3), and C600/pETS7/pMMB-dscFimH (FimD_{His} + dscFimH) (lane 4). (B to D) Coomassie blue-stained SDS-polyacrylamide gel (B), Western blot developed with anti-FimCH antiserum (C), and Western blot developed with anti-HA antiserum (D) of outer membrane preparations after nickel chromatography from the strains C600/pCD/pBad18-Kn (FimC + FimD_{His}) (lanes 1), C600/pCD/pBad-FimH (FimC + FimD_{His} + FimH) (lanes 2), C600/pCD/p601 (FimC + FimD_{His} + FimH_R) (lanes 3), C600/pCD/p421 (FimC + FimD_{His} + FimH_P) (lanes 4), and C600/pCD/pMC10 (FimC + FimD_{His} + CsgE-HA) (lanes 5).

strand exchange. dscFimG was created by fusing the N-terminal extension of FimF, the putative subunit neighboring FimG, onto the C terminus of FimG (Fig. 1). dscFimG was stable in the periplasm in the absence of FimC and did not form a complex with FimC (data not shown). To determine if dscFimG was properly folded, its ability to interact with FimH in vitro was tested. To establish this assay, we first incubated purified FimC_{His}-FimG complexes with pure FimH (obtained from a FimC-FimH complex). FimH copurified with the Fim-

C_{His}-FimG complex after nickel chromatography, indicating the formation of FimC_{His}-FimG-FimH complexes (Fig. 3A, lane 1). Subunit-subunit complexes are stable in SDS at 25°C, but chaperone-subunit complexes are not (40, 46). FimG-FimH complexes remained stable in SDS at 25°C (Fig. 3A, lane 2) (46) but dissociated after incubation in SDS at 95°C (Fig. 3A, lane 1). FimC_{His}-(FimG)₂-FimH complexes were also detected (Fig. 3A, lane 2). Incubation of pure dscFimG (Fig. 3A, lane 3) with pure FimH resulted in the formation of dscFimG-FimH complexes that were stable in SDS at 25°C but dissociated at 95°C (Fig. 3A, lanes 5 and 4, respectively). This reaction did not result in the formation of (dscFimG)₂-FimH complexes, suggesting that dscFimG does not form multimers, presumably because its groove is capped by the added donor strand. Taken together, these results demonstrate that dscFimG is stable in the periplasm and properly folded.

The in vitro reconstitution experiments described above prove that dscFimG interacts with FimH. Thus, we next investigated the ability of dscFimG to be incorporated into the growing pilus. Previously, it was shown that assembly intermediates can be purified in a complex with FimD_{His} by nickel chromatography. Thus, coexpression of FimC, FimG, and FimH with FimD_{His} resulted in the formation of a FimC-FimG-FimH-FimD_{His} complex that can be purified by nickel chromatography (41) (Fig. 3B to D, lanes 2). The ability of dscFimG to dissociate FimC from the FimC-FimH-FimD complex and form a dscFimG-FimH-FimD complex was analyzed to determine if a subunit, in the absence of the chaperone, could allow donor strand exchange to occur. dscFimG did not carry out donor strand exchange with the FimC-FimH-FimD ternary complex, and thus, a dscFimG-FimH-FimD complex was not formed (Fig. 3B to D, lanes 3). These results argue that the chaperone, in an incoming chaperone-subunit complex, plays a critical role in the donor strand exchange reaction.

Type 1 pilus assembly can be monitored by hemagglutination. FimG-FimH tip fibrillae assemble on the bacterial surface when coexpressed with FimD and FimC, resulting in HA-positive cells (Fig. 3D) (41). However, replacing FimG with dscFimG resulted in HA-negative bacteria (Fig. 3D), confirming the copurification experiments showing that dscFimG is not incorporated into the growing pilus and supporting the conclusion that the chaperone is needed for donor strand exchange to occur.

Genetically linked FimGH does not produce functional pili.

Other bacterial adhesins, such as invasins of *Yersinia pseudotuberculosis* (14) and intimin of enteropathogenic *E. coli* (4, 23, 31), are single proteins composed of multiple Ig domains, with a specialized adhesive domain at their end. This is similar to the pilus, which is also comprised of multiple Ig domains, with an adhesin at its tip. However, in the pilus, each Ig domain is a separate protein with an incomplete Ig fold that is completed by its neighboring subunit (40). A FimG-FimH fusion protein (FimGH) (Fig. 1) was constructed to investigate the role of individual subunits in comprising a pilus. The *fimG* gene was fused onto the 3' end of *fimH*, so that the N-terminal extension of FimG should complete the Ig fold of the FimH pilin domain in the fusion protein. However, the FimG pilin would still require FimC for stability. The production of FimGH in the periplasm in the absence and presence of FimC was monitored. FimGH was stable in the periplasm only when FimC was

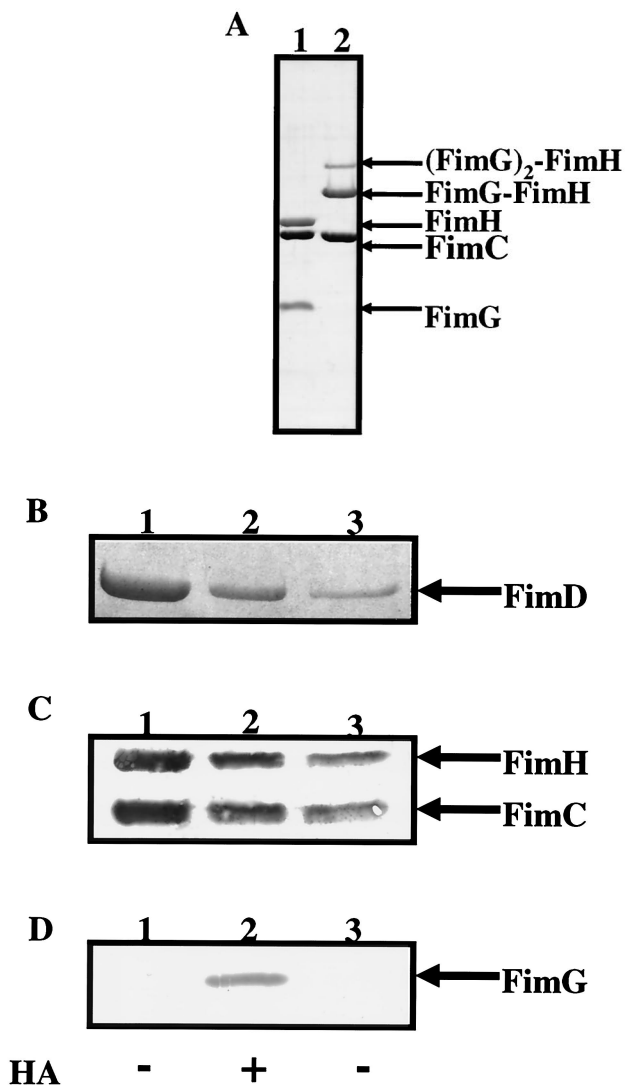


FIG. 3. dscFimG is not incorporated into a growing pilus. (A) Coomassie blue-stained gel of FimC_{His}-FimG-FimH complexes incubated at 95°C (lane 1) or 25°C (lane 2), purified dscFimG (lane 3), or dscFimG-FimH complexes incubated at 95°C (lane 4) or 25°C (lane 5). (B to D) Coomassie blue-stained gel (B), Western blot developed with anti-FimCH antiserum (C), or Western blot developed with anti-FimG antiserum (D) of outer membrane preparations after nickel chromatography from the strains C600/pCD-Kn/pMB7/pTrc99A (FimC + FimD_{His} + FimH) (lanes 1), C600/CD-Kn/pMB7/pTrc-FimG (FimC + FimD_{His} + FimH + FimG) (lanes 2), and C600/pCD-Kn/pMB7/pTrc-dscFimG (FimC + FimD_{His} + FimH + dscFimG) (lanes 3). The ability of the strains to hemagglutinate (HA) guinea pig red blood cells is indicated at the bottom of panel D (+, HA; -, no HA).

coexpressed (Fig. 4A, lanes 3 and 4). Additionally, FimGH bound to mannose and formed a complex with FimC, indicating that it was properly folded (data not shown).

Next, FimGH binding to FimD was assessed to determine if FimGH was targeted for pilus assembly. FimC-FimGH complexes copurified with FimD_{His} by nickel chromatography (Fig. 4B and C, lanes 1), indicating that FimGH was appropriately targeted to FimD for pilus assembly. Hemagglutination assays were performed to determine if FimGH is incorporated into the pilus. For strains Orn103/pUT2002/pTrc99A, Orn103/pUT2002/pTrc-FimH, and Orn103/pUT2002/pTrc-FimGH, the HA titers were 0, 32, and 0, respectively. Plasmid pUT2002 encodes a *fimH* mutant type 1 gene cluster, and strain Orn103 does not produce type 1 pili. Thus, the coexpression of FimGH with a *fimH* mutant type 1 gene cluster resulted in HA-negative bacteria, while coexpression of FimH restored the HA titer. We wanted to determine why FimGH did not produce functional pili. After FimC-FimGH binds to FimD, the next step in the biogenesis pathway is the binding of the next chaperone-subunit complex to the FimC-FimGH-FimD complex, which would be either FimC-FimF or FimC-FimG. Thus, the ability

of either FimG or FimF to copurify with FimC-FimGH-FimD complexes was assayed. Both FimG and FimF copurified with FimC-FimGH-FimD_{His} complexes (Fig. 4B to E, lanes 2 and 3), indicating that FimGH formed appropriate interactions with other pilus subunits. Even though FimGH was properly targeted for pilus assembly and interacted with other pilus subunits (Fig. 4), functional type 1 pili were not produced when FimGH was coexpressed with a *fimH* mutant type 1 gene cluster (above). Thus, we conclude that the donor strand exchange reaction between individual subunits provides the fiber with the necessary steric properties to be translocated through the usher. The FimGH fusion, while properly folded and capable of targeting to the usher and participating in donor strand exchange with incoming FimC-FimG and/or FimC-FimF complexes, may be unable to be translocated across the usher. An alternative hypothesis is that FimGH is translocated across the usher but the FimGH fusion protein in the pilus is unable to bind mannose. We favor the first hypothesis because FimH in the FimGH fusion protein is capable of binding mannose. When coexpressed with FimC, the FimGH fusion protein can be purified from the periplasm by using mannose Sepharose beads (data not shown).

DISCUSSION

Pilus assembly is thought to occur via a donor strand exchange reaction at the usher, where the G1β strand of the chaperone, occupying the groove of a subunit, is exchanged for the N-terminal extension of an incoming and neighboring subunit (8, 38, 40). During type 1 pilus biogenesis, FimH is the first

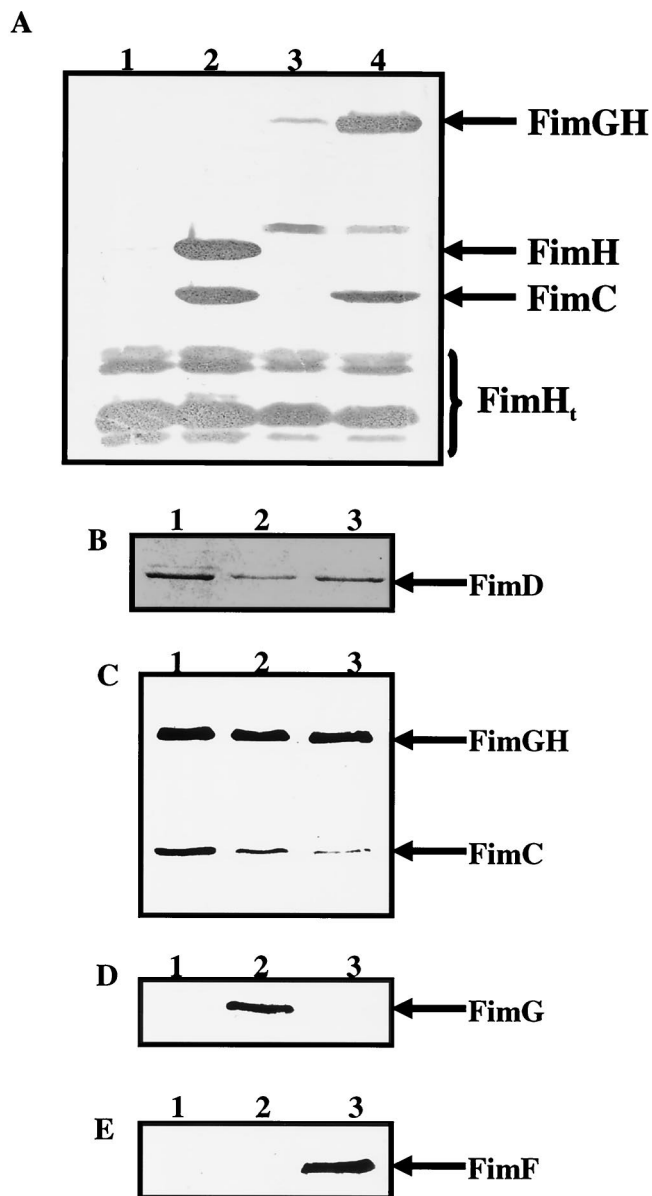


FIG. 4. FimGH is stable in the periplasm and is targeted for pilus assembly. (A) Western blot of periplasms prepared from strains C600/pMMB-FimH (FimH) (lane 1), C600/pHJ9205/pMMB-FimH (FimC + FimH) (lane 2), C600/pMMB-FimGH (FimGH) (lane 3), and C600/pHJ9205/pMMB-FimGH (FimC + FimGH) (lane 4). The Western blot was developed with anti-FimCH antiserum. (B to E) Coomassie blue-stained SDS-polyacrylamide gel (B), Western blot developed with anti-FimCH antiserum (C), Western blot developed with anti-FimG antiserum (D), or Western blot developed with anti-FimF antiserum (E) of outer membrane preparations after nickel chromatography from strains C600/pCD-Kn/pMB10/pTrc99A (FimC + FimD_{His} + FimGH) (lanes 1), C600/pCD-Kn/pMB10/pTrc-FimG (FimC + FimD_{His} + FimGH + FimG) (lanes 2), and C600/pCD-Kn/pMB10/pTrc-FimF (FimC + FimD_{His} + FimGH + FimF) (lanes 3).

protein to be incorporated into the growing pilus (10, 42). The absence of FimH results in a severe assembly defect, suggesting that the FimH-FimD interaction plays a critical role in initiating pilus assembly (9, 28). The next event in the biogenesis cascade is the targeting of the FimC-FimG complex to the

FimC-FimH-FimD ternary complex where donor strand exchange occurs. This results in the dissociation of the G1 β strand of FimC from FimH in the FimC-FimH-FimD complex and the replacement of this strand by the N-terminal extension of FimG (3, 8, 38, 41). We used the type 1 pilus assembly system as a model to investigate the mechanistic details and structural requirements of donor strand exchange.

The role of the adhesin in initiating pilus assembly suggests that it contains structural properties distinct from those of the other subunits. The FimC-FimH complex is in fact the only chaperone-subunit complex to bind strongly to the usher (42). We hypothesized that the unique information must therefore reside in the FimH protein itself. Thus, we used dscFimH to test this hypothesis. dscFimH does not require FimC for folding, since it contains its naturally missing β strand (as a C-terminal fusion) that is otherwise transiently provided by FimC during folding and the N-terminal extension of FimG during pilus assembly (3). We discovered that dscFimH was able to target to FimD and form a stable complex (Fig. 2A). We interpreted this result as indicating that FimH has sufficient information necessary for FimD binding. Structurally, the only difference between FimH and other pilus subunits is the presence of an additional domain, the N-terminal receptor binding domain, suggesting that this is the domain that interacts with FimD. However, we went on to show that both the receptor binding and pilin domains of FimH bound to FimD (Fig. 2B to D). Interestingly, other pilus subunits, like FimG or FimF, do not bind well to FimD (41, 42), suggesting that the pilin domain of FimH has unique properties enabling it to interact

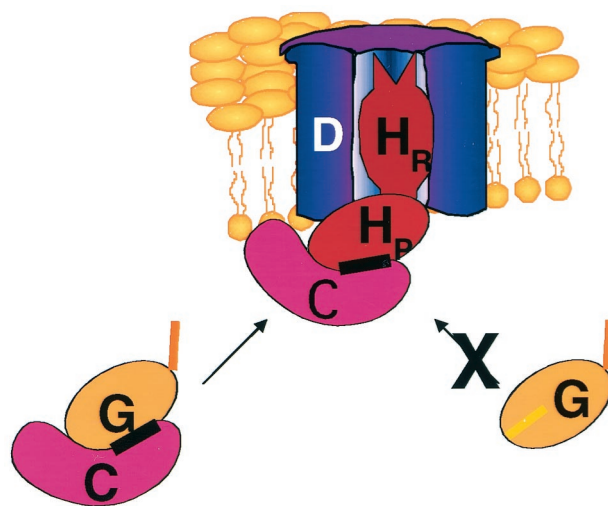


FIG. 5. Model of type 1 pilus assembly at the usher. The FimC-FimH complex binds to the FimD usher, inducing a conformation change in FimD (42), presumably into an assembly-competent state. We hypothesize that the FimH receptor binding domain (H_R) is inserted into the FimD pore, allowing the FimH pilin domain (H_P) to be exposed on the periplasmic side of FimD to remain available for donor strand exchange with FimC-FimG complexes. dscFimG was not incorporated into the growing pilus, suggesting that the chaperone is required for appropriate targeting and assembly of subunits into the pilus. The black line on FimC represents the FimC G1 β strand completing the Ig fold of either FimG or FimH. The yellow line of dscFimG represents the N-terminal extension of FimF completing the Ig fold of FimG.

with FimD. Since FimH is L shaped, we propose that the receptor binding domain is inserted into the channel of FimD, allowing the simultaneous docking of the pilin domain of FimH to the periplasmic surface of FimD (Fig. 5). The interactive groove of FimH occupied by the FimC chaperone is presumably exposed to the periplasm in order to properly orient it for participation in a donor strand exchange reaction with an incoming chaperone-subunit complex.

The efficient targeting of all subsequent chaperone-subunit complexes to the FimD usher (and their subsequent incorporation into the pilus) requires the presence of FimH to make the initiating interactions described above. Thus, FimC-FimH forms a ternary complex with FimD (41, 42), which is primed to participate in a donor strand exchange reaction with an incoming FimC-FimG complex. The N-terminal extension of FimG is exposed in the FimC-FimG complex. It is thus presumably free to exchange with the G1 β strand of the chaperone to facilitate chaperone dissociation (38) and the assembly of FimG to the pilin domain of FimH. We investigated the requirement for the FimC chaperone in the FimC-FimG complex to carry out this reaction by examining the ability of dscFimG to be incorporated into the growing pilus. dscFimG was shown to have all of the information necessary to bind and assemble to FimH in an *in vitro* reconstitution assay (Fig. 3A). However, *in vivo*, dscFimG was not able to dissociate FimC from the FimC-FimH-FimD ternary complex and it was unable to form a dscFimG-FimH-FimD complex (Fig. 3B to D and Fig. 5). The best interpretation of this result is that the donor strand exchange reaction requires the presence of the chaperone bound to an incoming subunit. This is consistent with previous work demonstrating that the chaperone is required for pilus assembly at a step after chaperone-adhesin binding to the usher (19).

FimGH was created to investigate the requirement of individual subunits in comprising a growing pilus. FimGH was functional in every aspect of pilus assembly tested except for its ability to be translocated across the outer membrane usher. It bound to the FimD usher and participated in donor strand exchange assembly reactions with incoming FimC-FimG and FimC-FimF complexes (Fig. 4). Yet, it did not produce functional pili when coexpressed with a *fimH* mutant type 1 gene cluster (see above). Instead, expression of FimGH blocked pilus assembly presumably by jamming the usher and preventing outward growth of the fiber. Thus, individual subunits assembled via donor strand exchange may provide the needed steric freedom to traverse the FimD pore.

The results presented here provide new insights into the assembly of pili via the chaperone-usher pathway. We have demonstrated that the receptor binding domain of FimH probably makes the first interaction with FimD, presumably being inserted into the pore, allowing the FimH pilin domain (bound to the chaperone) to lock onto the periplasmic surface of FimD and become properly oriented for donor strand exchange with incoming FimC-FimG complexes (Fig. 5). With the exception of chaperone-adhesin complexes, all other chaperone-subunit complexes bind poorly or not at all to the usher (10, 42). However, when the usher has been primed by an interaction with FimH, proper targeting and assembly of all other chaperone-subunit complexes occur. It is well established that the chaperone is critical for appropriate pilus assembly because it

is required to facilitate subunit folding and to prevent nonproductive aggregation (3, 17, 21, 30, 39). However, here we have shown that the chaperone also plays a critical role at the usher during donor strand exchange. It is required to facilitate the necessary interactions at the usher site for pilus assembly to occur. The finding that the targeting and donor strand exchange reactions during pilus assembly depend on properties of the chaperone opens up new insights and paves the way for future studies investigating the mechanistic details of the donor strand exchange reaction at the outer membrane usher.

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