Adaptor function of PapF depends on donor strand exchange in P-pilus biogenesis of Escherichia coli

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Adaptor Function of PapF Depends on Donor Strand Exchange in P-Pilus Biogenesis of Escherichia coli

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P-pilus biogenesis occurs via the highly conserved chaperone-usher pathway and involves the strict coordination of multiple subunit proteins. All nonadhesive structural P-pilus subunits possess the same topology, consisting of two domains: an incomplete immunoglobulin-like fold (pilin body) and an N-terminal extension. Pilus subunits form interactions with one another through donor strand exchange, occurring at the usher, in which the N-terminal extension of an incoming subunit completes the pilin body of the preceding subunit, allowing the incorporation of the subunit into the pilus fiber. In this study, pilus subunits in which the N-terminal extension was either deleted or swapped with that of another subunit were used to examine the role of each domain of PapF in functions involving donor strand exchange and hierarchical assembly. We found that the N-terminal extension of PapF is required to adapt the PapG adhesin to the tip of the fiber. The pilin body of PapF is required to efficiently initiate assembly of the remainder of the pilus, with the assistance of the N-terminal extension. Thus, distinct functions were assigned to each region of the PapF subunit. In conclusion, all pilin subunits possess the same overall architectural topology; however, each N-terminal extension and pilin body has specific functions in pilus biogenesis.

P pili are adhesive organelles that are critical virulence factors in the establishment of pyelonephritis by uropathogenic Escherichia coli (23, 36–38). These adhesive organelles are macromolecular, fibrous structures on the surface of the bacteria that mediate the recognition of and attachment to tissues of the kidney by the pathogen (5, 17, 23, 29, 31, 34, 36–38, 42). This attachment is a key step in the pathogenic cascade of uropathogenic E. coli, leading to the establishment of infection (36, 38). Assembly of pilus fibers requires the coordination of at least 11 structural and regulatory genes contained in the pap gene cluster (33, 35) and proceeds via the chaperone-usher pathway (43). This pathway is utilized by a wide variety of gram-negative bacteria to produce more than 100 different adhesive organelles, of which the P pilus is considered a prototypical member (15, 19; unpublished data).

P pili have a multicomponent architecture, consisting of a thin tip fibrillum and a thicker cylindrical rod that are each composed of multiple subunits (PapG, PapF, PapE, PapK, and PapA) (Fig. 1A) (2, 5–7, 11, 13, 21, 23, 25). Pili are assembled in a top-down fashion as they emerge from the bacterial cell surface (43), and the adhesin PapG is localized to the distal tip of the fiber (Fig. 1A) (25, 31). The order of the remaining pilus subunits has been delineated through a combination of biochemical studies and electron microscopy (2, 6, 7, 13, 21, 23, 25). The tip fibrillum is thought to be composed of PapG, PapF, and PapE (Fig. 1A) (21, 25, 32). It is a flexible structure consisting mainly of repeating PapE subunits interacting linearly with each other to form an approximately 2-nm-diameter polymer (21, 25). PapF is thought to connect PapG to the PapE polymer (21, 32, 34), and the entire tip fibrillum is connected via the PapK adaptor protein (21) to the rigid pilus rod consisting of PapA subunits (2) (Fig. 1A). The thick rod contains PapA subunits arranged to form a right-handed helical cylinder with 3.28 subunits per turn (2, 7, 13). Finally, the PapH subunit is thought to terminate assembly and anchor the entire composite structure to the outer membrane (1) (Fig. 1A).

Each nonadhesin subunit in the pilus fiber contains two domains, each of which has the same overall topology in all subunits: an N-terminal extension and an incomplete immunoglobulin (Ig)-like fold (pilin body) (39, 40) (Fig. 1A). Instead of an N-terminal extension, the adhesin subunit has a receptor-binding domain connected to the pilin body domain (9, 11). The N-terminal extensions each contain a conserved motif of alternating hydrophobic residues, including an invariant glycine (39, 40) (Fig. 1B). A conserved cysteine demarcates the boundary between this domain and the first A1 beta strand of the pilin body (39, 40) (Fig. 1B). The pilin body of each subunit lacks the final seventh beta strand of the canonical Ig fold (3, 9, 39, 40). The missing strand exposes the hydrophobic core and renders each of the subunits highly unstable, targeting it for rapid proteolytic degradation in the periplasmic space if it is not coproduced with the PapD chaperone (3, 9, 22, 28, 39, 40). The chaperone consists of two complete Ig-like domains (3, 9, 14, 18–20, 27, 39, 40). In order to stabilize the subunits, the chaperone donates the G1 beta strand to complete the fold of the subunit, inserting parallel to the F beta strand of the pilin body in donor strand complementation (3, 9, 39, 40).

In donor strand exchange, the N-terminal extension of an incoming pilus subunit displaces the G1 beta strand of PapD...
from a neighboring subunit (3, 4, 9, 39, 40) and inserts antiparallel to the subunit C-terminal F beta strand, creating a stable canonical Ig-like fold (9, 39, 40). These tight interactions are thought to give the fiber its extraordinary stability (9, 39, 40). The process of donor strand exchange depends on the PapC usher (40). The usher is a twin-pore complex with 2- to 3-nm channels (30, 44). Each of the chaperone-subunit complexes is targeted in turn to the usher, beginning with the PapD-PapG chaperone-adhesin complex, which has the highest affinity for PapC (10, 41, 44). Following binding to the usher, the chaperone in each complex dissociates from the pilus subunit (18), exposing the underlying interactive surfaces of the subunit (26, 40) and allowing donor strand exchange to proceed (4, 9, 18, 39, 40).

Each subunit has the same overall topology and undergoes the same assembly processes via the chaperone-usher pathway as the other subunits (9, 39, 40), leading to speculation that each subunit is functionally equivalent to the others. However, recent investigations into the activation of the CpxRA two-component pathway by P-pilus assembly revealed that various subunits each have distinct activities (28). Furthermore, early characterization of PapF suggested that it has unique adaptor and initiator roles in pilus biogenesis (21, 32). E. coli K-12 strains carrying a papF mutant operon, constructed from the E. coli J96 pap operon, exhibited severe pilus assembly defects compared to strains carrying a wild-type pap operon (21). This phenotype was found to be unique to strains with mutations in papF (21). Strains deficient in PapF also showed an inability to agglutinate human red blood cells, an activity mediated by the PapG adhesin (21). The basis for these properties of PapF remains elusive.

In this study, the correlation between the arrangement and function of subunits in the pilus fiber was investigated. N-terminal deleted (Ntd) and N-terminal swapped (Nts) subunits were used to examine and alter the order of subunit incorporation in the fibers. We found that the PapF N-terminal extension was necessary for PapF to function as the PapG adaptor protein, while the specific PapF pilin body was not required for interactions with PapG. In contrast, the specific PapF pilin body was necessary for PapF to interact with PapE, as well as to actively participate in the initiation of the assembly of the remainder of the pilus structure. These studies demonstrate that each domain of each subunit is distinct from the others in function. Thus, characterization of the role of PapF in the prototypical P-pilus system may provide insight into assembly events that may also occur in the biogenesis of a multitude of other adhesive fibers.

**MATERIALS AND METHODS**

**Bacterial strains and genetic constructs.** All bacterial strains used in this study are listed in Table 1. E. coli laboratory strain MC4100 (8), which does not carry the pap operon, was used as the host strain in all studies except in cloning steps. Cloning and subcloning were done with E. coli laboratory strains MC1061 (45) and TOP10 (Invitrogen).

Plasmid pYML76 carrying the papE mutant operon under control of the natural promoter was made by moving the EcoRI-BamH fragment encoding the papE mutant operon from pPAP15 (33) into pACYC184 (NEB). A blunt end was generated at the BamH site of the papE mutant fragment prior to ligation into a pACYC184 vector that was cut with EcoRI and Scal. The following plasmids have been previously described elsewhere. Plasmid pPAP15 contains a papE mutant operon in which papE was disrupted by insertion of an XhoI linker (33). pF3J contains the pap operon under control of the native promoter in
TABLE 1. Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td>Strains</td>
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<tr>
<td>MC4100</td>
<td>F araD139 Δ(argF-lac)U169 rpsL150 (Streptomyces) relA1 fliB5301 deoC1 ptsF25 rbsR</td>
<td>8</td>
</tr>
<tr>
<td>MC1061</td>
<td>F araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lacX74 rpsL (Streptomyces) hsdR2 (rK K- mK K- mCra mCra mB1)</td>
<td>45</td>
</tr>
<tr>
<td>TOP10</td>
<td>F mcrA Δ(mrr-hsdRMS-mcrBC) Invitrogen</td>
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<tr>
<td>Plasmids</td>
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</tr>
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<tr>
<td>pACYC184</td>
<td>Expression vector</td>
<td>NEB</td>
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<td>pPAPl5</td>
<td>papeE mutant operon</td>
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</tr>
<tr>
<td>pFJ9</td>
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<tr>
<td>pHJ13</td>
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<td>pYML36</td>
<td>NtdPapE expression plasmid</td>
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<td>pYML43</td>
<td>Wild-type PapF expression plasmid</td>
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<tr>
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<td>NtdPapF expression plasmid</td>
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<td>pYML75</td>
<td>NtsPapK expression plasmid</td>
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<tr>
<td>pYML76</td>
<td>papeE mutant operon (natural promoter)</td>
<td>This study</td>
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</table>


HA assays. Hemagglutination (HA) assays were performed essentially as previously described (21), except that pilus production was induced by one growth passage instead of three. This was done to inhibit accumulation of mutants or revertants due to the overproduction of wild-type (e.g., PapE) or mutant subunits. Briefly, a starter bacterial culture was grown to saturation overnight in LB broth in the presence of antibiotics appropriate for the strain. A 0.5 ml portion of the saturated overnight culture was then spread on large petri dishes of LB broth in the presence of antibiotics appropriate for the strain. A 500-μl volume of the saturated overnight culture was then spread on large petri dishes containing solid tryptic soy agar (for induction of pili from the bacterial cells were then scraped from the plates, weighed, and resuspended in 1× PBS (5 ml per g of cells). Separate aliquots of each cell suspension were then subjected to either isolation of the pili or extraction of the periplasmic contents. For pilus preparations, resuspended cells were heated at 65°C for 1 h to release P pili (21). Depleted cells were then pelleted by centrifugation, and the resultant crude pilus preparations were incubated in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer supplemented with 4 M urea at 95°C for 5 min prior to analysis by SDS-PAGE followed by Coomassie blue staining or by immunoblotting using anti-PapG pilin antibodies in conjunction with anti-rabbit immunoglobulin G-alkaline phosphatase conjugate secondary antibodies. Immunoblots were developed using the 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)-nitroblue tetrazolium substrate system (Sigma). Periplasm was prepared by the lysozyme-EDTA method as previously described (10) and subjected to immunoblotting as described above.

**RESULTS**

The PapF N-terminal extension is required for proper PapG presentation. Jacob-Dubuisson et al. demonstrated that PapF is required for E. coli expressing P pili to agglutinate human red blood cells (21), presumably as a result of its role in adapting PapG to the remainder of the pili fiber. To understand the molecular basis for the function of PapF as an adaptor protein, the ability of Ntd and Nts (Fig. 1C) PapF and PapE subunits to complement a pap gene cluster lacking papF in HA assays was tested. Ntd subunits lack the majority of the amino-terminal extensions but retain the signal sequences and pilin bodies (Fig. 1C) (28, 40). In Nts subunits, the complete N-terminal extension of one subunit is exchanged with that of another subunit, without modification of the pilin body of the subunit (Fig. 1C) (28). The Ntd and Nts constructs therefore enable examination of each specific domain of the pili subunits (the N-terminal extension or the pilin body domains) in functional assays of pilus biogenesis, such as HA assays. E. coli cells expressing pili from the wild-type pap operon are able to agglutinate human red blood cells (18, 21). Agglutination results from the interaction of the PapG adhesin with moieties on the red blood cells, and thus, the HA assay provides a measure of the correct incorporation and proper presentation of PapG at the end of the tip fibrillum (18, 21). Therefore, Ntd and Nts subunits were used as tools in HA assays to determine whether the presentation of PapG could be altered by the presence or absence of the specific PapF N-terminal extension or pilin body.

In these assays, E. coli laboratory strain MC4100 was used because the pap operon is not present in the genome of this bacterium. A PapF− strain was constructed by transforming pFJ9, a plasmid with a papF mutant pap operon under control of the natural promoter (21), into MC4100. Wild-type PapF, wild-type PapE, NtdPapF, NtsPapE, NtsPapF, or an empty vector (pMMB66) was then supplied in trans, and the strains were tested to determine their abilities to agglutinate red blood cells. MC4100 carrying the entire wild-type pap operon and MC4100 carrying an empty vector (pACYC184) were also examined to establish producing and nonproducing levels of HA titers for comparison in each experiment. E. coli MC4100 cells carrying the wild-type pap operon produced a...
positive HA titer, while cells with only the empty vector gave no titer (Fig. 2A). Similar to the no-operon vector control, PapF− bacteria to which the parent vector pMMB66 was added were also unable to agglutinate red blood cells, yielding HA titers of 0 (Fig. 2A). These results are consistent with PapF− studies conducted previously in our laboratory (21). In contrast, PapF− strains complemented with wild-type PapF demonstrated a restored ability to haemagglutinate (Fig. 2A). Positive HA correlated with the presence of PapGI in the pilus fibers, as determined by immunoblot analysis using anti-PapGI pilin antibody (Fig. 2B). While PapG was found in the periplasm of the PapF− strain carrying an empty vector, it was not observed in the pilus fiber (Fig. 2B). In contrast, abundant PapG was observed in the periplasm and pilus fiber upon complementation of the PapF− strain with PapF (Fig. 2B).

If PapF follows PapG in the mature pilus fiber, then the PapF N-terminal extension should be required to complete the Ig-like fold of the PapG pilin body in donor strand exchange, covering the hydrophobic groove caused by the absence of the final beta strand in PapG and consequently enabling proper presentation for PapG-mediated agglutination of red blood cells (Fig. 2C). To test the involvement of the specific N-terminal extension of PapF in the interaction with PapG, the levels of HA in response to production of NtsFPapE in the PapF− strain were analyzed. Similar to strains with wild-type PapF, strains complemented with NtsFPapE, which carries the N-terminal extension of PapF on the pilin body of PapE, re-
sulted in the presence of PapG in pilus preparations and exhibited positive HA (Fig. 2A and 2B). To determine if the presence of the PapE pilin body also contributed to the ability of NtsE/PapE to interact with PapG, two PapE subunits, wild-type PapE and Ntd/PapE, were tested. Both subunits were unable to complement the PapF− strain in HA assays (Fig. 2A), and no PapG was observed in pilus preparations when an immunoblot assay was performed (Fig. 2B). Consistent with these results, replacement of only the PapF N-terminal extension with that of PapE on the pilin body of PapF, to form NtsE/PapF, did not yield a subunit capable of substituting for wild-type PapF in the HA assay or in localization of PapG in pili, despite normal levels of PapG in the periplasm (Fig. 2A and 2B). Production of this NtsE/PapF protein in the PapF− strain resulted in a titer of 0 (Fig. 2A), despite the presence of the PapF pilin body. Likewise, removal of the PapF N-terminal extension, leaving only the pilin body of PapF (Ntd/PapF), resulted in an inability of the new subunit to complement the PapF− strain, and an HA titer of 0 was observed (Fig. 2A), showing that the PapF Ig-like fold could not establish interactions with PapG in the absence of the PapF N-terminal extension. These results suggest both that the N-terminal extension of PapE does not fit into the hydrophobic groove of the PapG pilin body and that the pilin body of PapF is not required in interactions between PapF and PapG (Fig. 2C). Taken together, the results of these PapF− complementation studies show that the adaptor function of PapF is dependent upon the N-terminal extension of PapF and not the specific pilin body of PapF.

The N-terminal extension mediates specificity and facilitates subunit arrangement via donor strand exchange. In the PapF− studies described above, the adaptor function of PapF was demonstrated to be dependent on the specific PapF N-terminal extension through its participation in interactions with the pilin body of PapG via donor strand exchange, facilitating the proper arrangement of the two subunits in the pilus fiber (Fig. 2C). Therefore, if PapF connects PapG to the rest of the pilus, then rather than participation in interactions with PapG, the requirement for the pilin body of PapF in pilus biogenesis may be to form specific interactions with the N-terminal extension of PapE (Fig. 2C). In turn, the PapE pilin body would form interactions with the N-terminal extension of another PapE subunit or of a PapK subunit; PapK would then interact with PapA, resulting in the previously described order of subunits. To investigate the role of donor strand exchange in mediating the arrangement of all of the subunits in the pilus, Ntd and Nts subunits were produced in a PapE− strain, and the abilities of the resultant bacteria to agglutinate red blood cells were monitored. The PapE− strain was constructed like the PapF− strain, by transforming a plasmid, pYM176, containing a papE mutant operon under control of the natural promoter (33), into E. coli MC4100. Wild-type PapE, wild-type PapF, Ntd/PapE, Ntd/PapF, NtsE/PapE, NtsE/PapF, Nts/PapK, or the empty vector (pMMB66) was then added in trans. PapE− strains to which pMMB66 was added were unable to agglutinate red blood cells, yielding HA titers of 0 (Table 2), consistent with PapE− studies conducted previously (21). In contrast, PapE− strains complemented with wild-type PapE showed positive HA titers (Table 2).

Previous studies have placed repeating subunits of PapE between PapF and PapK in the final pilus architecture (Fig. 1A) (21, 25). To determine the requirement for the PapE N-terminal extension for connecting PapF to the rest of the pilus fiber, the levels of HA in response to production of NtsE/PapF or NtsE/PapK in the PapE− strain were analyzed. NtsE/PapF was unable to restore HA ability to the PapE− strain (Table 2). However, NtsE/PapK was able to complement the PapE− strain, such that HA titers equivalent to those of strains with the wild-type pap operon were observed (Table 2). NtsE/PapF and NtsE/PapK both carry the PapE N-terminal extension, but they differ in the pilin body, suggesting that while the N-terminal extension of PapE could interact with a preceding PapF subunit, the difference in the abilities to complement the PapE− strain resulted from the difference in the pilin body. The failure of NtsE/PapF to bridge PapF to PapK therefore suggests that the PapF pilin body could not accommodate the N-terminal extension of an incoming PapK subunit. In contrast, the PapK pilin body of NtsE/PapK was able to interact with the N-terminal extension of an incoming PapA subunit. These results directly confirm previously reported orders of subunits in the mature pilus fiber and demonstrate the requirement for specific N-terminal extensions in mediating specificity between subunit-subunit interactions.

Complementation of the PapE− strain by NtsE/PapK and not by NtsE/PapF demonstrated the specificities of interactions between PapF and PapE and between PapK and PapA, resulting in the proper arrangement of subunits in the pilus (Fig. 1A). To investigate the requirement for the PapE pilin body in mediating interactions between PapE and PapK through donor strand exchange, NtsE/PapE was tested in the HA assay. NtsE/PapE was able to complement the PapE− strain, producing positive HA titers (Table 2). The presence of the N-terminal extension of PapF in NtsE/PapE suggests either that PapF subunits may have some ability to interact with one another or, more likely, that overexpression of NtsE/PapE from the Plac promoter allowed it to outcompete native PapF produced from the natural promoter in the papE mutant operon for interactions with PapG. Irrespective of these possibilities, these results also show that interactions between PapE and PapK required the PapE pilin body, placing PapE before PapK in the pilus fiber, as previously described. Finally, Ntd/PapE, wild-type PapF, and Ntd/PapF were also examined. Ntd/PapE, wild-type PapF, and Ntd/PapF were each unable to confer the ability to agglutinate red blood cells to the PapE− strain (Table 2). Taken together, these results suggest that the N-terminal extension facilitates the

<table>
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<th>TABLE 2. HA in PapE− strains</th>
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<tr>
<td>Strain</td>
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<tr>
<td>Wild-type pap operon (pF3)</td>
</tr>
<tr>
<td>pACYC184 vector (no operon)</td>
</tr>
<tr>
<td>PapE−/pMMB66</td>
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<tr>
<td>PapE−/pPapE</td>
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<tr>
<td>PapE−/NtsE/PapE</td>
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<td>PapE−/NtsE/PapK</td>
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<td>PapE−/Ntd/PapE</td>
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<td>PapE−/PapF</td>
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<tr>
<td>PapE−/Ntd/PapF</td>
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*HA titers are expressed as the highest dilution of the cell suspension that produced detectable HA. The HA titers are the averages for three experiments.
PapF is necessary for nucleation of P-pilus assembly. The HA studies described above demonstrated that the PapF N-terminal extension is necessary for PapF to function as a PapG adaptor, dictating the arrangement of subunits through donor strand exchange. It has also been previously noted that PapF may play a role in the initiation of assembly of the remaining subunits into the fiber (21). The basis for this initiator function is unclear. To directly examine the involvement of the N-terminal extension and the pilin body in the initiation of pilus assembly by PapF, relative piliation levels of PapF- strains were compared when wild-type or mutant PapF and PapE subunits were expressed in trans. As in the HA assays, strains MC4100/pFJ3 (wild-type pap operon) and MC4100/pACYC184 (empty vector) were used as positive and negative controls, respectively, for comparison. Piliation in populations of 200 bacterial cells per strain was characterized and quantitated using negative-stain electron microscopy. In addition, levels of piliation were also demonstrated by visualization of PapA via SDS-PAGE of pilus preparations followed by Coomassie blue staining.

Cells carrying the wild-type pap operon produced abundant pili, with the majority of the bacterial cells showing moderate to high levels of piliation (Fig. 2A and 3A, panel I). Cells with only the empty vector were bald and devoid of pili (Fig. 2A and 3A, panel II). PapF- strains to which pMMB66 was added exhibited a strong defect in pilus assembly, producing low levels of pili or no pili (Fig. 2A and 3A, panel III). As shown in Fig. 2A and 3A, panel IV, in PapF- strains complemented with wild-type PapF, piliation was restored to wild-type (i.e., whole pap operon) levels, with pili abundant on the cell surface. Consistent with these electron microscopy results, the levels of PapA in pili isolated from these PapF- strains were higher in the presence of PapF than in the absence of PapF, similar to results obtained for control strains carrying the wild-type pap operon or no operon (Fig. 3B).

To determine if the specific N-terminal extension of PapF was necessary for nucleation of pilus assembly, piliation was assessed by electron microscopy and isolation of pili in the PapF- strain after addition of the subunit NtsFPapE. In the HA assays described above, NtsFPapE was able to restore the ability to hemagglutinate in the PapF- strain (Fig. 2A). In contrast, the same subunit, NtsPapE, was not able to restore piliation to wild-type levels in the PapF- strain, and only a small number of cells exhibited moderate to high levels of pili (Fig. 2A and 3A, panel V). Likewise, wild-type PapE and NtdPapE controls yielded mostly cells with low numbers of pili or no pili (Fig. 2A and 3A, panels VI and VII). In agreement with the electron microscopy results, only small amounts of PapA were observed in pili prepared from all three of these strains (Fig. 3B). These results suggest that while the specific PapF N-terminal extension was required to interact with PapG, it alone was not specifically responsible for PapF’s role as an initiator of pilus assembly.

If the specific N-terminal extension of PapF was not required for initiation of pilus assembly, the specific pilin body may be necessary for this function. Thus, piliation was measured in PapF- strains producing NtsFPapF or NtdPapF, two proteins which contain the same PapF pilin body but differ by the presence or absence of an N-terminal extension. The presence of the N-terminal extension of PapE on the PapF pilin body (NtsFPapF) increased the levels of piliation in PapF- strains, enabling more than one-half of the observed PapF- cells to produce abundant pili (Fig. 2A and 3A, panel VIII). Likewise, high levels of PapA were also determined by SDS-PAGE of pili prepared from the PapF-/NtsFPapF strains (Fig. 3B). Lower levels of piliation were observed in PapF- strains to which NtdPapF was added (Fig. 2A, 3A, panel IX,
and 3B). Notably, however, these results are in contrast to the results of the HA studies described above, as neither NtdPapF nor NtsE_papF was able to complement the PapF− strain in HA assays (Fig. 2A), consistent with the requirement of the specific PapF N-terminal extension to adapt PapG to the pilus. These results show that the specific pilin body of PapF was essential to initiate pilus assembly and that both a PapF N-terminal extension and a PapE N-terminal extension could facilitate this function.

**DISCUSSION**

Assembly of P pili occurs via the chaperone-usher pathway, a highly conserved pathway that is used in the construction of more than 100 other adhesive organelles in a wide range of diverse gram-negative bacteria (15, 19; unpublished data). The P pilus is a prototypical example of these various adhesive organelles (16, 43). All structural subunits in P pili share the same overall topology (39, 40). Thus, the subunits could possibly all have the same unilateral function; i.e., they could act as simple building blocks within the final pilus fiber. However, through examination of the PapF structure and function, we report in this study a more complex role of the different pilus subunits in pilus biogenesis and show that there may be significant differences in function between structurally similar subunits.

In this work, Ntd and Nts subunits were used to demonstrate that the ordering of the subunit types in the pilus is determined by the donor strand exchange reaction involving specific N-terminal extensions. The Ntd and Nts subunits were used because they enabled examination of each domain of the pilus subunits: the N-terminal extension and the pilin body. We found that in a PapF− strain, the N-terminal extension of PapF was required for adapting PapG to the pilus tip. As shown in experiments with NtsE_papE, even in the absence of the PapF pilin body, PapG was adapted to the pilus tip as long as the PapF N-terminal extension was presented. Since PapG is the first subunit incorporated into a growing pilus at the outer membrane usher, the N-terminal extension of PapF must interact with the pilin domain of PapG, presumably by completing the Ig-like fold during donor strand exchange. Consistent with these results was the inability of the NtsE_papF subunit to adapt PapG to the pilus tip, as determined in the HA assay. These results show directly that the ability of PapF to interact specifically with PapG is dependent only on the N-terminal extension.

In order to complement a PapE− strain and restore HA titers, a minimum of two requirements must be met: (i) an N-terminal extension must be available for interaction with the pilin body of PapF and (ii) an appropriate pilin body must be present for interaction with the N-terminal extension of an incoming pilus subunit. The subunits NtsE_papE and NtsE_papK both fulfilled each of these requirements. NtsE_papE, carrying the PapF N-terminal extension, presumably competes with wild-type PapF for interaction with PapG and, in contrast to PapF, possesses the PapE Ig-like domain, enabling it to interact with the appropriate incoming subunit. Restoration of HA titers in the PapE− strain by NtsE_papK was allowed because the presence of the N-terminal extension of PapE permitted NtsE_papK to interact with PapF, while simultaneous interaction with PapA was possible due to the presence of the pilin body of PapK. These experiments thus confirm the following order for pilus subunit incorporation into the fiber, mediated by the N-terminal extensions: PapG, PapF, PapE, PapK, PapA (Fig. 1A).

It has been previously shown that papF mutants produce few or no pili, suggesting that PapF may also act as an initiator of pilus assembly (21). We discovered that the adaptor function of PapF, mediated by the N-terminal extension, could not fully account for this putative initiator function. Further, while the specific N-terminal extension of PapF is not required for this initiator function, the pilin body of PapF must be presented in the context of an N-terminal extension for efficient initiation, as reflected by the highly diminished ability of NtdPapF to promote pilus assembly. How PapF controls pilus assembly is currently not understood; however, it may aid the usher PapC in its transition into an assembly-competent state. Saulino et al. demonstrated a shift in the usher from a closed state to an assembly-competent state in the related fim system upon binding to the adhesin, which in the fim system also serves as an initiator of pilus assembly (41). In the fim system, binding of all nonadhesin chaperone-subunit complexes depended on the initial binding of the FimH adhesin-chaperone complex, which induced the usher shift, possibly explaining the additional role of FimH in initiation of pilus assembly (41). A similar shift has not been reported for PapC in the P-pilus system, possibly because PapF may assist the adhesin in this process. Unlike in the fim system, in the P-pilus system, usher binding by the subunit-chaperone complex that immediately follows the adhesin, PapF-PapD, is not dependent on prior binding by the PapG-PapD adhesin-chaperone complex (10). Instead, PapF-PapD complexes, like PapG-PapD adhesin-chaperone complexes, have been shown to independently bind to the usher in vitro assays (10). We thus argue that in addition to events that may be mediated by PapG, the initiation event in P-pilus biogenesis also requires an interaction with PapC involving the PapF pilin body.

In conclusion, by using a combination of Ntd and Nts subunits, we were able to dissect the adaptor and nucleator functions of PapF and assign separate domains of the protein to the different functions. We discovered that the specific PapF N-terminal extension played an important role in interactions with PapG, while at the same time, it facilitated the ability of the Ig-like domain to initiate pilus assembly. The pilin body likewise had dual functions in the cell, working in part to facilitate pilus assembly by playing an active role in the biogenesis pathway, while at the same time providing an interactive surface for binding with subsequent subunits.

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REFERENCES


