Assembly of Complex Organelles: Pilus Biogenesis in Gram-Negative Bacteria as a Model System

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Pathogenic bacteria assemble a variety of adhesive structures on their surface for attachment to host cells. Some of these structures are quite complex. For example, the hair-like organelles known as pili or fimbriae are generally composed of several components and often exhibit composite morphologies. In Gram-negative bacteria assembly of pili requires that the subunits cross the cytoplasmic membrane, fold correctly in the periplasm, target to the outer membrane, assemble into an ordered structure, and cross the outer membrane to the cell surface. Thus, pilus biogenesis provides a model for a number of basic biological problems including protein folding, trafficking, secretion, and the ordered assembly of proteins into complex structures. P pilus biogenesis represents one of the best-understood pilus systems. P pili are produced by 80–90% of all pyelonephritic Escherichia coli and are a major virulence determinant for urinary tract infections. Two specialized assembly factors known as the periplasmic chaperone and outer membrane usher are required for P pilus assembly. A chaperone/usher pathway is now known to be required for the biogenesis of more than 30 different adhesive structures in diverse Gram-negative pathogenic bacteria. Elucidation of the chaperone/usher pathway was brought about through a powerful combination of molecular, biochemical, and biophysical techniques. This review discusses these approaches as they relate to pilus assembly, with an emphasis on newer techniques. 

A key step in the pathogenesis of an organism is recognition of and attachment to its host cell. Gram-negative bacteria assemble a number of adhesive organelles designed for this purpose, including pili or fimbriae. Pili are hair-like structures that radiate out from the bacterial surface (Fig. 1) and present adhesive moieties known as adhesins that recognize specific host cell receptors (1). This binding event allows a bacterium to establish contact with its host and initiate events leading to either a commensal or a pathogenic relationship. Consistent with this role, pili are significant virulence factors associated with a wide range of diseases.

Adhesive pili are assembled by at least three distinct pathways. Biogenesis of a superfamily of more than 30 pili and nonpilus adhesins requires outer membrane proteins known as ushers that work together with periplasmic chaperones as part of the chaperone/usher pathway (1, 2). P pili represent the prototypic organelle assembled by the chaperone/usher pathway. P pilus are composite structures, consisting of a thin, flexible tip fibrillum connected to a rigid helical rod (Figs. 1 and 2) (3, 4). The tip fibrillum is composed mainly of repeating subunits of the PapE protein arranged in an open-helical fiber 2 nm in diameter. The pilus rod is a right-handed helix 6.8 nm in diameter formed by repeating subunits of PapA. The PapG adhesin is situated at the distal end of the tip fibrillum. PapG binds to Galα(1–4)Gal epitopes present in the globo series of glycolipids found in the human kidney (5). Consistent with this binding specificity, P pilus are important virulence determinants for the development of pyelonephritis (6). The PapF and PapK proteins are present in very low copy number in the pilus and are proposed to function as initiators and/or adaptors for pilus assembly (4, 7). PapF is thought to link the PapG adhesin to the tip fibrillum and PapK is thought to link the tip fibrillum to the PapA rod. The PapH protein, also present in very low copy number, acts to terminate the rod and may serve as a pilus anchor in the outer membrane (8).
The periplasmic chaperone for P pili is PapD. PapD facilitates release of pilus subunits from the cytoplasmic membrane into the periplasm and is required for their proper folding in the periplasm (Fig. 2) (9). The chaperone caps interactive surfaces on the subunits, preventing their premature aggregation in the periplasm (10). In the absence of the chaperone, subunits form aggregates that are degraded by the DegP periplasmic protease (9). PapD recognizes a conserved carboxyl-terminal tail present on each of the pilus subunits (11). Recent crystal structures (12a, 12b) have revealed that PapD binding serves to shield an exposed region of the subunit's hydrophobic core, completing the structure of the subunit through a mechanism termed donor strand complementation. The bound carboxyl terminus of the subunit also forms part of an interactive surface that participates in subunit-subunit interactions subsequent to chaperone dissociation (12c, 13). Thus, by binding to this region the chaperone prevents subunits from interacting prematurely in the periplasm.

Periplasmic PapD-subunit complexes are targeted to the PapC usher in the outer membrane (Fig. 2) (14). Interaction of a chaperone-subunit complex with the usher triggers dissociation of the chaperone from the subunit by an unknown mechanism. This results in exposure of subunit assembly surfaces previously capped by the chaperone. The exposed interactive surfaces subsequently go on to participate in energetically more favorable subunit-subunit interactions, driving their incorporation into the growing pilus fiber. The assembly and translocation of the pilus fiber across the outer membrane depends on the PapC usher. PapC is an integral outer membrane protein that was recently shown to form oligomeric rings containing a central channel (15). In the absence of the usher, chaperone-subunit complexes accumulate in the periplasm but...
pili do not appear on the cell surface (16, 17). Thus, the usher likely serves as a channel for translocation of pili to the cell surface.

A second pathway for assembling adhesive pili is exemplified by type 4 or bundle-forming pili. These are long, polarly localized pili expressed by a number of Gram-negative bacteria that are responsible for twitching motility and bacterial aggregation as well as adhesion (18, 19). Type 4 pili assembly occurs independent of a chaperone/usher pathway and requires 14 or more assembly components (20, 21). Type 4 pilus biogenesis shares many similarities with the main terminal branch of the general secretory pathway or type II secretion (22). Both systems express a number of proteins with homology to type 4 pilus subunits. These proteins are thought to organize into some sort of supramolecular structure involved in secretion (21, 23). In addition, the assembly of this putative structure depends on proteins that are also required for pilus assembly. Translocation of type 4 pili to the cell surface requires an outer membrane protein termed a secretin that forms large oligomeric rings with apparent central pores (24). The type II secretory pathway also requires a secretin for export of its substrates. The secretin presumably plays a role analogous to that of the usher but these two protein families share no sequence homology.

A third assembly pathway is used to assemble fibers known as curli or thin aggregative pili (25). Curli are thin adhesive filaments expressed by Escherichia coli and Salmonella that coil up into a tangled mass on the bacterial surface. These organelles are thought to

**FIG. 2.** Model for the biogenesis of P pili. Pilus subunits (PapG, F, E, K, A, H) are translocated across the cytoplasmic membrane by the Sec general secretory machinery. The subunits interact sequentially with the periplasmic disulfide isomerase DsbA and the periplasmic chaperone PapD. DsbA is required for proper disulfide bond formation in the subunits as well as in PapD. DsbA is also required for the correct folding of PapD (85). PapD facilitates release of pilus subunits from the cytoplasmic membrane and participates in proper folding of the subunits through a mechanism termed donor strand complementation. PapD binds to and caps interactive surfaces present on the subunits. In the absence of PapD, subunits enter into off-pathway interactions leading to aggregation and eventual degradation by the periplasmic DegP protease. Off-pathway subunit interactions are sensed by the Cpx two-component signal transduction pathway. The Cpx pathway activates a number of genes including degP and also affects pap expression. Cpx likely affects the expression of other virulence factors as well. Chaperone-subunit complexes in the periplasm are targeted to the PapC usher in the outer membrane. Interaction with the usher leads to uncapping of the chaperone from the subunit, allowing subunit-subunit interactions that lead to the formation and translocation of a linear pilus fiber across the outer membrane through the usher channel. The diameter of the usher channel would constrain the pilus rod to a linear conformation while in the usher channel. The rod would then adopt its final helical conformation on reaching the cell surface. Coiling of the rod outside the cell may facilitate the outward growth of pili.
assemble extracellularly through a nucleation/prep- 
cipitation mechanism (26). The main curli sub-
unit, CsgA, has been shown to be secreted outside 
the bacterium in a soluble form. Assembly of CsgA into 
fibers requires the CsgB nucleator protein, which is 
thought to initiate precipitation and assembly of CsgA 
on the cell surface. A CsgA `CsgB` strain will secrete 
CsgA subunits that can be assembled into curli on the 
surface of a CsgA `CsgB` recipient strain (26). Thus, 
CsgB appears to act extracellularly. Homologs to the 
periplasmic chaperone and outer membrane usher are 
not present in the curli assembly pathway. The mecha-
nism by which the subunits reach the cell surface is 
currently unknown.

Assembly mechanisms for adhesive pili in addition 
to the three described above are likely to exist. CS1 and 
related pili expressed by enterotoxigenic E. coli (ETEC) 
have no homology to any of the above systems. Four 
genes, cooA through cooD, are required for CS1 pilus 
assembly (27). CooA is the major pilus subunit and 
CooD is a minor subunit present at the tip of the pilus. 
The CooB protein is not part of the final structure, but 
was recently shown to form periplasmic complexes 
with both CooA and CooD and protect them from degrada-
tion (28). This is very reminiscent of the action of 
the PapD chaperone. The CooC protein is an integral 
outer membrane protein that could function as an 
usher. Thus, a unique chaperone/usher-like pathway 
may assemble CS1 pili.

Pilus assembly touches on a number of basic biolog-
ical questions. For example, how do proteins fold into 
domains that serve as modules for assembling complex 
organelles? How are the proteins targeted to the proper 
place at the proper time and assembled in the correct 
order? Pilus biogenesis also relates to the broader prob-
lem of how Gram-negative bacteria secrete proteins 
across their outer membrane. This review discusses 
the approaches used to elucidate pilus biogenesis, fo-
cusing on the chaperone/usher pathway. A review of 
techniques for studying pilus by Kuehn et al. appeared 
several years ago (29). This review touches on some of 
the methods covered in that article but focuses on 
advances since that time. Readers are referred to the 
et earlier review for additional information.

IDENTIFICATION OF PILUS GENES

Nearly all pili are coded for by genes clustered at a 
single region of the chromosome or on virulence plas-
mids. The gene cluster coding for P pilus, termed pap for 
pyelonephritis-associated pili, was cloned from an E. 
coli urinary tract isolate by Hull et al. (30). The pap 
locus contains 11 genes (Fig. 1). The two regulatory 
genres, papI and papB, are located upstream from the 
structural and assembly components, which are orga-
nized in a single operon (1). Chaperone/usher-
dependent pili tend to have similarly organized gene 
custers. For example, the type 1 (fim) locus has an 
an organization nearly identical to that of the pap locus 
(Fig. 1). Type 1 pili are expressed throughout the En-
terobacteriaceae (31). The type 1 adhesin, FimH, binds 
to glycoproteins present in the bladder epithelium and 
a number of other tissues in a mannose-sensitive man-
ner (1). In both the fim and pap systems, the gene 
coding for the major pilus subunit is found at the 
beginning of the operon, just downstream of the regu-
ulatory genes. The genes for the minor pilus subunits 
are located at the distal end of the operon, with the 
adhesin last, and the genes coding for the chaperone 
and usher are located in the middle. Both the fim and 
pap loci were identified by screening a cosmid library 
for the ability to confer agglutination and pilus assem-
bl'y on a nonpiliated laboratory strain (30). Laboratory 
and nonpathogenic strains often lack virulence loci 
such as pili. One can take advantage of this in screens 
such as that used to identify the pap locus or through 
use of subtractive techniques to identify regions unique 
to the pathogenic bacteria that are likely to play a role 
in virulence and may contain pilus gene clusters (32).

Searching for homology in gene and protein data-
banks (such as the GenBank sequence database: 
www.ncbi.nlm.nih.gov) can help identify pilus genes 
and their potential functions. Pilus structural subunits 
typically contain identifying conserved features. All 
pilus subunits assembled by PapD-like chaperones 
contain a highly conserved carboxyl-terminal motif 
characterized by a series of alternating hydrophobic 
residues flanked by a glycine located 14 residues from 
the carboxyl terminus and a penultimate tyrosine (11). 
These subunits also contain a second conserved motif 
located near the amino terminus (13) as well as two 
conserved cysteines spaced approximately 30 residues 
apart (33). Pilus assembly components are usually 
highly conserved. In the chaperone/usher superfamily, 
the periplasmic chaperones exhibit 25–56% amino acid 
identity (34). The outer membrane usher proteins also 
share this high level of identity (29). With the com-
pleted and ongoing sequencing of bacterial genomes 
(32) many new genes with homology to pilus compo-
nents are likely to be identified. The E. coli K12 ge-
nome alone contains at least three chaperone/usher 
pairs of unknown function (2).

FUNCTION OF PILUS GENES

Once a gene cluster coding for a pilus has been 
dcloned, a number of techniques are available for deter-
mining the function of individual genes. A first step is 
to place the pilus operon under control of an inducible 
promoter, as turning on pilus expression from the nat-
ural promoter often requires specific and time-consuming conditions. The requirement for a gene and the role of its product can be probed using transposon or linker insertions, gene deletions, frameshift mutations, and cloning of individual genes onto plasmids (29). Minicell analysis has been used successfully in the past to identify products of particular genes (29). Mutant gene clusters can be assayed for the ability to assemble adhesive pili. Adhesion is most easily tested using hemagglutination assays (HA). These assays take advantage of the ability of most pili to bind to specific carbohydrate moieties present on red blood cells. Bacteria expressing pili crosslink or agglutinate red blood cells via multiple adhesin–receptor interactions. Hemagglutination titers can be measured by serially diluting bacteria in microtiter wells to determine the minimum dilution of bacteria that can still cause hemagglutination (29). The carbohydrate receptor for an adhesin can be investigated by screening erythrocytes deficient in specific blood group antigens for agglutination or by assaying various glycosidic enzymes for their ability to inhibit HA (29). Overlay assays can be performed in which piliated bacteria are tested for binding to glycolipids that have been separated using thin-layer chromatography (35). Mutating the gene encoding the adhesin will abolish the ability of the bacteria to mediate the specific HA reaction being studied. However, mutations in genes required for the assembly of pili will result in the same HA-negative phenotype. In addition, mutations in genes that encode adaptors that join the adhesin to the pilus fiber will also reduce or abolish the assembly of adhesive pili. Thus, it is often difficult to prove which gene encodes the adhesin relying solely on genetics. One genetic method that was successful was used in the identification of the PapG adhesin. PapG was identified by a transcomplementation experiment using a related pilus gene cluster called prs. A mutation in prsG abolished the ability of the bacteria to bind to the Forssman antigen, which is present on sheep erythrocytes but not human erythrocytes. PapG complemented this mutation but shifted the binding specificity to the related globoside receptor that is present on human erythrocytes. Thus, incorporation of PapG into the Prs pilus changed the binding specificity and HA profiles, which was genetic proof that PapG encoded an adhesin (36). However, the unlikely possibility that PapG was necessary for the expression or modification of the true adhesin could not be ruled out. Thus, formal proof requires a biochemical analysis showing that the purified protein binds to the respective receptor (see below).

The major pilus subunit can be identified by purifying pili and visualizing the major band by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (29). Pili are typically purified using a blender to shear off the pilus or by heat treatment. The puriﬁed pili are then harvested by precipitation with magnesium, which crosslinks the negatively charged pilus rods, or by sucrose gradient centrifugation (29). The protein–protein interactions in pili can be quite strong and resistant even to boiling in SDS. Addition of urea or acid is often necessary to dissociate pili completely. Dissociation of P pili for visualization of the PapA monomer by SDS–PAGE requires addition of 4 M urea to the sample buffer. Minor pilus subunits often cannot be detected by SDS–PAGE due to their low abundance. In this case, antibodies against the minor proteins can be used to identify and localize minor pilus subunits using immunoblots of pure pili preparations and immunogold electron microscopy (29, 37). High-resolution electron microscopy is necessary to provide images of the architecture of mutant and wild-type pili. The composite nature of P pili (4) was identiﬁed using a quick-freeze deep-etch electron microscopy technique (Fig. 1) (38). High-resolution electron microscopy is useful for determining the contribution of various subunits to the molecular architecture of a pilus by determining the effect of gene knockouts or overexpression on pilus phenotype. For example, expression of a papK pap operon resulted in the production of longer than normal tip ﬁbrillae, suggesting that PapK may regulate tip length (4). Overproduction of PapK in the presence of a wild-type pap operon caused a shift to signiﬁcantly shorter tip ﬁbrillae, conﬁrming the length-modulatory role of PapK (7). Furthermore, expression of PapK was shown to facilitate assembly of the PapA rod. These experiments demonstrated that incorporation of PapK into the tip ﬁbrillum terminates growth of the tip ﬁber and that PapK functions as an adaptor for initiation of PapA rod assembly.

PILUS BIOGENESIS IN GRAM-NEGATIVE BACTERIA

Expression of pilus subunits in the absence of the chaperone typically results in formation of aggregates in the periplasm that are degraded by the DegP protease (9). These aggregates can be toxic to the host in the absence of DegP. Obviously, this creates difficulties for the purification of pilus subunits. However, coexpression of the chaperone can alleviate this problem. Subunits that aggregate when expressed alone can be purified from the periplasm as complexes with the periplasmic chaperone. High-level protein expression is achieved by placing the chaperone and subunit under the control of an inducible promoter(s) such as tac, ara, or lac. Stable chaperone–subunit complexes form and accumulate in the periplasm. Periplasms are harvested by converting the bacteria to spheroplasts using lysozyme/EDTA treatment (29). The spheroplasts are then pelleted and the periplasmic contents collected as the supernatant fraction. Previously, purification of
the PapD chaperone was performed using cation-exchange chromatography, taking advantage of the high pI of the PapD family of chaperones (29). Purification of chaperone–subunit complexes has been done using a variety of chromatographic steps including ion-exchange and hydrophobic interaction columns (29). A simpler method is to insert a polyhistidine tag (or any other convenient affinity tag) onto the carboxyl terminus of the chaperone and use nickel affinity resin to bind the chaperone or chaperone–subunit complexes from the periplasm (Fig. 3). Functionality of the His-tagged chaperone can be tested by its ability to complement a chaperone minus operon for assembly of adhesive pili as determined by HA.

Purification of His-Tagged Chaperone–Subunit Complexes from the Periplasm

The following procedure allows purification of His-tagged PapD or chaperone–subunit complexes from the periplasm. Prepare periplasmic extracts from bacteria induced for expression of the His-tagged chaperone or chaperone–subunit complexes. Prepare periplasmic extracts from bacteria induced for expression of the His-tagged chaperone or chaperone–subunit complexes (Fig. 3). Dialyze the periplasmic extracts against buffer [we typically use 20 mM Tris (pH 8.0)] to remove the EDTA, which will interfere with binding to the nickel beads. The dialysis and subsequent steps should be carried out at 4°C or on ice, unless noted otherwise. For “batch” purification, prepare affinity resin such as Ni²⁺-nitrilotriacetic acid (NTA) (Qiagen, Valencia, CA) or Talon Metal Affinity Resin (Clontech, Palo Alto, CA) by washing two or three times with buffer and resuspending to a final concentration of 50% (v/v) in buffer. Add 0.5 ml of the washed beads to periplasmic extracts harvested from 0.5 g wet wt cells (~50 ml of culture) and rock the mixture gently for 30 to 60 min at room temperature. Wash the bound beads at least three times with 10 times the bead volume. Addition of salt and low concentrations of imidazole (10–20 mM) can help wash off contaminants. Elute the chaperone–subunit complex(es) by rocking two to four times (10 min each, room temperature) in 4 times the bead volume with buffer containing 250 mM imidazole or 20 to 100 mM EDTA. The eluate should contain highly purified chaperone or chaperone–subunit complexes (Fig. 3). Dialyze the eluate to remove the imidazole or EDTA if either one will interfere with subsequent assays. Experience with Pap and type 1 complexes indicates that some of the chaperone–subunit complexes are susceptible to dissociation and/or precipitation on freezing and thawing. Therefore, the stability of a particular chaperone–subunit complex to various storage conditions should be determined. We often store the complexes on ice for use soon after purification. A drawback to purification using a His-tagged chaperone is that free chaperone is always present in excess in the periplasm, and, in addition to purifying the chaperone–subunit complex, an overabundance of free chaperone is also purified. An additional round of purification is needed to separate the free chaperone from the chaperone–subunit complexes. Typically, this can be achieved by hydrophobic interaction chromatography (29, 39).

Native gel electrophoresis is very useful for assaying chaperone–subunit complex formation (29). PapD and related chaperones generally have a high pI (greater than 9) and run at a characteristic position on isoelectric focusing (IEF) and native PAGE. Binding of a subunit to the chaperone results in a shift to a lower pI (Pap subunits range in pI from 4.9 to 8.9). This allows determination of the relative amounts of free protein versus chaperone–subunit complexes. In addition, the band corresponding to the chaperone–subunit complex can be excised and run on a denaturing gel to determine the ratio of chaperone to subunit in the complex (10). We have found the PhastSystem (Pharmacia, Piscataway, N J) to be very convenient for IEF and native PAGE analysis of chaperone–subunit complexes (for native PAGE of basic proteins such as the chaperone a reversed-polarity electrode assembly is required). There are alternative strategies for purifying chaperone–subunit complexes that eliminate the problem of copurifying excess free chaperone in the first step. These affinity chromatography strategies center on using the subunit rather than the chaperone as a purification handle. In these protocols, free chaperone will not bind to the affinity matrix. Instead, the subunit binds to the affinity matrix as a chaperone–
subunit complex. Purification of a chaperone-adenosine complex can be carried out by receptor affinity chromatography if the receptor recognized by the adenosine is known and can be coupled to a matrix. For example, PapD–PapG complexes are routinely purified using Galα(1–4)Gal–Sepharose affinity chromatography (Fig. 3) (29). A soluble Galα(1–4)Gal synthetic receptor analog with high affinity for the PapG adhesin is then used to elute the PapD–PapG complexes. Recent work suggests that chaperone–subunit complexes can be purified using affinity chromatography by engineering a histidine tag onto the subunit instead of the chaperone (C. Liu, C. H. Jones, and S.J.H., unpublished data). This requires that the tag be placed at the amineterminus because the carboxyterminal serves as the recognition site for the chaperone. Success has been achieved by placing the histidines immediately before the start of the mature protein and inserting two to four alanines between the histidines and the signal sequence cleavage site. Placing the histidines directly following the cleavage site appears to block processing by the signal peptidase.

The usher proteins for P and type 1 pili have also been purified using a histidine tag (15, 40). However, purification of the usher requires special techniques because it is an integral outer membrane protein. The outer membrane must first be isolated and then treated with a detergent to extract the usher out of the membrane. A number of techniques are available for purifying outer membranes (41). An easy and efficient technique is to use differences in the composition of the cytoplasmic and outer membranes to selectively solubilize away the cytoplasmic membrane.

Purification of His-Tagged Usher from the Outer Membrane

The following procedure has been used to purify His-tagged PapC and FimD ushers from strains harboring the usher on a plasmid with an inducible promoter (15). Grow the bacteria in LB broth with aeration and induce for expression of the usher. Induction is typically initiated at mid-log phase of cell growth and continued for 1 h. Overexpression of membrane proteins can be harmful to the host. Growth curves should be done to determine the maximum usher expression the bacteria can tolerate without affecting growth rate. Harvest the bacteria, wash with 20 mM Tris–HCl (pH 8), and resuspend into 60 ml of the same buffer (this procedure is for 1 liter of original culture and should be scaled accordingly for different volumes). All steps beginning with harvesting of the bacteria should be carried out on ice or at 4°C unless specified otherwise. Break the bacteria by single passage through a French pressure cell (SLM Instruments, Rochester, NY) at 14,000 psi. Sonication can be used instead to break open the bacteria, but we have found a French press to be more efficient and better able to handle large volumes. Addition of protease inhibitors [phenylmethylsulfonyl fluoride (PMSF) or a cocktail such as Complete Protease Inhibitor (Boehringer-Mannheim, Indianapolis, IN)] before breaking open the bacteria is advisable. Perform a low-speed spin (3000g, 15 min) to remove any unbroken cells, then spin at 100,000g for 1 h to pellet total membranes. Resuspend the membrane pellet in 20 mM Tris–HCl (pH 8). Membrane pellets are most easily resuspended by starting with a small volume of buffer and using a glass rod (or a Pasteur pipet fire-sealed at the end) to break up the pellet into the buffer. Then use a pipet to gently suck up and expel the solution until the pellet is evenly resuspended (avoid generation of air bubbles). Dilute the resuspended membranes to 57 ml with buffer. Add 3 ml of 10% N-lauroylsarcosine (Sarkosyl) and rock at room temperature for 5 min. Sarkosyl selectively solubilizes only the cytoplasmic membrane. Pellet the outer membrane by centrifuging 100,000g for 1 h. Resuspend the outer membrane pellet to 27 ml with 20 mM Tris–HCl (pH 8) + 0.15 M NaCl. Add 3 ml 10% Zwittergent 3-14 (Calbiochem, La Jolla, CA) and rock 30 min at room temperature to solubilize the usher. The majority of the usher is found in the soluble fraction following this step. Other non-denaturing detergents such as dodecyl maltoside and octyl glucoside may be used in place of Zwittergent 3-14, but any detergent should be tested for efficient extraction of the usher from the outer membrane as well as compatibility with the usher. Do a final high-speed centrifugation as above to pellet away insoluble material. For “batch” purification, dilute the OM extract with 20 mM Tris–HCl (pH 8)–0.15 M NaCl to reduce the Zwittergent 3-14 concentration to 0.1% and rock the solution with Ni-NTA resin (Qiagen) overnight at 4°C to bind the His-tagged usher. Wash the Ni–NTA resin several times with buffer A [20 mM Tris–HCl (pH 8), 0.1% Zwittergent 3-14, 0.3 M NaCl] containing 0–20 mM imidazole to remove contaminants, and then elute the usher off the resin in buffer A plus 30 mM EDTA. The eluate should contain highly purified usher protein. If needed, the purified usher can be concentrated using a centrifugal concentrator (Ultrafree, Millipore, Bedford, MA, or equivalent) and/or dialyzed to remove the EDTA and reduce the salt concentration (the protein tends to precipitate below 0.1 M NaCl). We typically use a final storage buffer of 20 mM Tris–HCl (pH 8), 0.1% Zwittergent 3-14, and 150 mM NaCl.

Molecular Mechanisms of Pilus Assembly: Chaperone–Subunit Interactions

Crystallization of the PapD periplasmic chaperone was a defining event in deciphering the molecular
mechanism of pilus assembly by the chaperone/usher pathway (42). The crystal structure revealed two β-sheet immunoglobulin-like domains oriented toward each other with an overall boomerang shape (Fig. 4). The three-dimensional structure of PapD was used to align the PapD family of periplasmic chaperones (34, 43). All of the more than 30 members of the periplasmic chaperone family have a conserved hydrophobic core that maintains the overall immunoglobulin-like features of the two domains. The chaperones also contain a number of highly conserved surface-exposed residues. The most highly conserved surface residues lie in the cleft of the molecule. The invariant Arg-8 and Lys-112 residues as well as conserved hydrophobic residues at the exposed edge of the G1β strand at positions 103, 105, and 107 form part of the subunit binding site in the cleft of the chaperone (Fig. 4). The mechanism of action of the chaperone was probed by site-directed mutagenesis of conserved residues (11, 44). For example, site-directed mutagenesis of the two invariant residues in the chaperone cleft, Arg-8 and Lys-112, abolished the ability of PapD to bind to subunits without affecting the overall PapD structure (1, 11, 44). This helped to identify the interactive surface of the chaperone that is required for subunit binding and pilus assembly. The site-directed PapD mutants were tested by several techniques (11, 44). Mutant papD genes were used to complement a papD- pap operon, and assembly of pili was examined by HA and pilus purification. The effect of PapD mutations on the ability of the chaperone to bind subunits was investigated by determining the formation and stability of chaperone-subunit complexes in the periplasm. Functional chaperone-subunit complex formation can be assayed simply by looking for the presence of subunits in the periplasm, as many subunits are degraded in the absence of a functional chaperone. Protease susceptibility of subunits provides a measure of chaperone function because properly folded subunits are less susceptible to proteases. Chaperone-subunit complex formation can be moni-

**FIG. 4.** Crystal structure of the PapD–PapK chaperone-subunit complex. PapD is shown in green as a ribbon diagram, with domain 1 on the left and domain 2 on the right. PapK is shown as a ribbon diagram in blue. The conserved carboxyl terminal motif of PapK is anchored in the cleft of the chaperone via hydrogen bonds of the final carboxylate group with the invariant Arg-8 and Lys-112 residues of PapD (shown as stick models in white). The carboxyl terminal motif of PapK forms a β zipper with the G1β strand of PapD via backbone hydrogen bonds and hydrophobic interactions with the conserved alternating hydrophobic residues of the G1β strand (Leu-103, Ile-105, and Leu-107; depicted as stick models in red). The G1β strand of PapD inserts into a groove of PapK, completing the immunoglobulin fold of the subunit. In addition, the conserved alternating hydrophobic residues of the G1β strand form part of the hydrophobic core of PapK (depicted as stick models in yellow). This interaction has been termed donor strand complementation. Subunit-subunit interactions likely occur through a similar interaction termed donor strand exchange.
to red in pulse–chase experiments by immunoprecipitation using an antibody against the subunit in question. Chaperone–subunit complex formation can also be monitored using native PAGE as described above.

Alignment of the PapD family of periplasmic chaperones also revealed important differences. The immunoglobulin-like superfamily of chaperones can be divided into two subfamilies based on structural differences conserved within each subgroup (34). The chaperone subgroups have differences in the conserved sheet of domain 1 and in the number of amino acids in the loop between β strands F1 and G1, termed the F1–G1 loop. Chaperones with a short F1–G1 loop have been denoted the FGS (F1–G1 short) subfamily and chaperones with a long F1–G1 loop have been denoted the FGL (F1–G1 long) subfamily. Interestingly, FGS chaperones (like PapD) specifically assemble pili while FGL chaperones specifically assemble non-pilus-associated adhesins. FGL chaperones contain the invariant Arg-8 and Lys-112 residues as well as the conserved alternating hydrophobic residues in the G1 β strand that are known to form a critical part of the subunit binding site of FGS chaperones. This conservation argues that FGS and FGL chaperones use a similar subunit binding paradigm. All subunits assembled by FGS chaperones contain the conserved carboxyl-terminal motif known as the β zippe (see below). Subunits assembled by FGL chaperones contain a variation of this β zipper motif. The variation of the β zipper motif may have important ramifications on the molecular architecture of the nonpilus fibers that are assembled by the FGL chaperones.

Another advance came with the cocrystallization of PapD with a peptide from PapG corresponding to the conserved carboxyl terminus of all the pilus subunits (11). This subunit motif is bounded by a conserved penultimate aromatic residue and a conserved glycine positioned 14 residues from the COOH terminus. Between these two residues is a conserved pattern of alternating hydrophobic amino acids. The PapG peptide bound in an extended conformation along the exposed G1 β strand of PapD, with its carboxyl terminus anchored in the of the two chaperone domains through hydrogen bonds with the invariant Arg-8 and Lys-112 residues (Fig. 4). Positioning of the peptide along the chaperone’s β strand was due mostly to backbone hydrogen bonds. The alternating hydrophobic residues of the peptide made hydrophobic interactions with corresponding conserved alternating hydrophobic residues on PapD. The peptide essentially extends the β-sheet structure of domain 1 of PapD by one additional strand and this interaction has been termed a β zipper.

Experimental evidence suggests that the conserved carboxyl-terminal subunit motif is important for subunit-subunit interactions as well as chaperone-subunit interactions. Using freeze-thaw conditions that facilitate dissociation of the chaperone, rod and tip subassemblies were reconstituted in vitro from purified PapD–PapA and PapD–PapE complexes, respectively (12c). This demonstrated that dissociation of the chaperone from complexes triggers self-assembly of the corresponding pilus fibers. However, the presence of excess PapD blocked formation of the fibers, arguing that PapD binds competitively to a self-associative surface of the subunit. To investigate the possibility that the conserved carboxyl-terminal motif forms part of this self-associative surface, site-directed mutagenesis was used to modify the pattern of alternating hydrophobic residues of the carboxyl terminus of the PapG adhesin (13). The effect of point mutations within this region with respect to PapG–PapF interactions in the mature pilus was measured by the relative temperature required for dissociation of PapG from PapF in purified pilus tips. The mutations led to reduced PapG–PapF complex stability (i.e., a lower temperature was required for the complete dissociation of the variant PapGs from PapF relative to the wild-type adhesin). These results suggested that the β zipper motif of PapG constitutes part of an assembly surface that interacts with PapF. Mutation of the carboxyl-terminal motif of PapG also significantly reduced its ability to bind to PapD as assayed by ELISA. These results argue that PapD binds directly to a surface on PapG that PapG uses to assemble to PapF in the tip fibrillum after chaperone uncapping.

Binding of the chaperone to the conserved carboxyl terminal subunit motif is important for additional aspects of chaperone function. In the absence of the chaperone, subunits were found to remain tethered to the cytoplasmic membrane and eventually form aggregates (9). An in vitro assay using spheroplasts was developed to test the ability of the PapD chaperone to facilitate release of subunits from the cytoplasmic membrane (9). For this assay, spheroplasts were prepared according to the method of Witholt et al. (45) from a strain harboring a plasmid carrying the PapG adhesin under control of an inducible promoter. The spheroplasts were then induced for PapG expression in the presence or absence of the purified PapD chaperone in the outside medium. The spheroplasts were removed by centrifugation and the presence of chaperone–adhesin complexes in the supernatant was determined by immunoprecipitation. PapD was able to release subunits into the medium, even if added as much as 30 min after induction of PapG. Mutation of the invariant Arg-8 peptide anchoring residue of PapD abolished this activity, indicating formation of the β zipper was necessary for subunit release. Furthermore, mutagenesis or deletion of the conserved carboxyl terminus of PapG allowed release of PapG into the medium independent of the presence of PapD.
of the chaperone, suggesting that the conserved carboxyl terminus mediates tethering of the subunit to the membrane. Site-directed mutagenesis studies have indicated that formation of the β zipper between the chaperone and subunit is also important for the ability of the chaperone to fold subunits (9). These studies were done using the PapG adhesin. PapG was isolated from the periplasm by immunoprecipitation, and affinity of the adhesin for its receptor was used as an indication that PapG had attained a properly folded state. Less than 10% of immunoprecipitable PapG expressed in the absence of the PapD chaperone was able to bind receptor. Coexpression of wild-type PapD, but not PapD mutated at the peptide anchoring Arg-8 or Lys-112 residues, allowed nearly 100% of immunoprecipitable PapG to bind to receptor. This implies that proper chaperone-subunit interaction is required for correct folding of the PapG adhesin in vivo.

Two recent crystal structures have placed the evidence for chaperone function described above into a complete structural context. The PapD–PapK chaperone-subunit complex from P pilus and the FimC–FimH chaperone–adhesin complex from type 1 pili have been crystallized (Fig. 4) (12a, 12b). The PapK subunit and the subunit domain of the FimH adhesin were revealed to have immunoglobulin folds similar to domain 1 of the chaperone. However, the subunits lack the seventh β strand present in canonical immunoglobulin folds. The absence of this strand produces a deep groove along the surface of the pilin domain, exposing its hydrophobic core. One edge of this groove is formed by the conserved carboxyl terminal motif of the subunit. In the crystal structure, binding of the chaperone to the conserved subunit motif results in the chaperone donating its G1 β strand to the subunit, completing the immunoglobulin fold of the subunit in a mechanism termed donor strand complementation (Fig. 4). This interaction stabilizes the subunit by shielding its hydrophobic core and explains the requirement of the chaperone for proper subunit folding and the instability of subunits when expressed without the chaperone. In addition, by binding to the conserved subunit motif the chaperone simultaneously caps one of the subunit’s interactive surfaces, preventing premature pilus formation in the periplasm. Experimental evidence also points to a second conserved assembly surface located at the subunit’s amino terminus (13). In the PapD–PapK crystal structure, this conserved amino-terminal region does not contribute to the immunoglobulin fold of the subunit but rather projects away from the rest of the subunit where it would be free to interact with another subunit. During pilus biogenesis at the usher, the amino terminal extension of one subunit may displace the donated chaperone β strand from its neighboring subunit in a mechanism termed donor strand exchange. The mature pilus would thus consist of an array of immunoglobulin domains, each of which contributes a strand to the fold of the preceding subunit to produce the organelle. As a whole, the above studies show how a combination of structural, modeling, site-directed mutagenesis and functional analyses has allowed insight into the molecular basis of how the chaperone controls the development of pilus fibers. Subsequent steps in assembly such as targeting to the usher and chaperone uncapping are not as well understood, but are under investigation using similar structure/function approaches.

Structures at atomic resolution provide invaluable information and crystal structures have driven much of the work and understanding of pilus assembly. Besides the P and type 1 crystal structures described above, the only other pilus structural protein crystallized to date is the type 4 pilin protein from Neisseria gonorrhoeae (46). This pilin was purified for crystallography by subjecting purified pilus to repeated cycles of disassembly and reassembly, followed by isoelectric focusing and gel filtration (47). The pilin was found to be modified by addition of an O-linked disaccharide and to contain a globular head with strikingly long α-helical spine. The type 4 pilin was modeled to pack into the pilus fiber to form a smooth cylinder with the long, hydrophobic α helices in the center and only carbohydrate and hypervariable regions exposed to solvent, providing a structural basis for the high antigenic variation displayed by type 4 pili. In lieu of a crystal structure, much structural information can still be obtained. As discussed above, electron microscopy has proved particularly useful for probing the structure of pili. Electron microscopy and fiber diffraction studies have been used to build a three-dimensional reconstruction of P pilus (3). This reconstruction revealed the diameters of the tip and rod (20 and 68 Å, respectively). Furthermore, the rod was shown to be formed by the winding of the linear PapA fiber into a right-handed helix containing 3.28 subunits per turn, with a helical cavity of 20 Å connected to the external environment by numerous radial channels.

### MOLECULAR MECHANISMS OF PILUS ASSEMBLY: USHER–CHAPERONE–SUBUNIT INTERACTIONS

Crystallization of the usher presents a special problem, as integral membrane proteins require the presence of detergents and tend to aggregate and precipitate during the crystallization process. Outer membrane proteins are particularly difficult to crystallize, with only a handful solved to date. A number of specialized techniques have been developed in attempts to solve membrane protein structures, such as two-dimensional electron crystallography and lipidic...
cubic phases (48, 49). Quick-freeze, deep-etch electron microscopy was used to gain structural information about the PapC usher protein (15). Purified PapC usher was found to assemble into ring-shaped complexes containing an apparent central pore (Fig. 5). The pore-forming ability of PapC was confirmed by a separate assay (see below). The pore of PapC measured 2–3 nm in diameter, which is big enough to allow passage of the 6.8-nm-diameter helical pilus rod. A solution to this problem was revealed, again using electron microscopy, by demonstrating that for P pili, as well as type 1 pili, the helical rod can be unraveled into linear fibers 2 nm in diameter (Fig. 5) (15). Thus, a model was proposed in which the pilus rod is constrained to a linear form as it traverses through the usher and only coils up into its final helical structure when it reaches the cell surface (Fig. 2). The chaperone/usher system lacks any obvious components for harnessing energy available at the cytoplasmic membrane and has been shown to function independently of cellular energy (50). Winding of the rod into a helix on the cell surface may help drive translocation of pili across the outer membrane.

A number of additional techniques have been used to probe the structure of the usher. Anti-PapC immunoblots of isolated outer membranes showed that the usher migrated as a high-molecular-weight species if not boiled, providing the first indication that PapC assembled into an oligomeric complex (15). Determining the number of PapC subunits present in the oligomer has proved to be difficult. Gel filtration is a standard technique used to determine protein size, but this is more complicated with membrane proteins as detergent effects must be taken into consideration. Subjecting both purified and membrane-bound PapC to crosslinking, for example, with bis(sulfosuccinimidyl)suberate (BS3, Pierce, Rockford, IL), revealed a ladder of high-molecular-weight crosslinked species. However, these bands were diffuse and difficult to interpret, likely due to intramolecular as well as intermolecular crosslinks. A native gel electrophoresis technique termed “Blue Native PAGE” (51) has yielded the best results for the stoichiometry of the PapC complex. Blue Native PAGE allows membrane proteins to be electrophoresed in the absence of detergent or crosslinker by using Coomassie blue G dye to keep the proteins soluble without denaturation or disruption of protein complexes. The dye also confers a negative charge on the proteins, which will migrate through the gel in response to an electric field and fractionate according to size. Examination of the PapC usher by Blue Native PAGE revealed that the PapC oligomer ran as a series of high-molecular-weight species (Fig. 5) (15). The band of slowest mobility corresponded to a molecular mass of ~550 kDa, consistent with a hexameric complex. The smaller-molecular-weight bands likely reflect dissociation states of the complex, as purification from the outer membrane partially destabilizes the PapC oligomer (15). The oligomeric complexes formed by the secretin family of outer membrane proteins are much more stable than the ushers, resistant even to boiling in SDS (24, 52). The secretins are thought to consist of 12–14 subunits, as estimated by techniques including low-percentage SDS–PAGE and scanning transmission electron microscopy (STEM) (24, 53, 54). STEM allows weight determination by comparison of a protein of interest with a standard of known molecular weight, usually tobacco mosaic virus.

**FIG. 5.** The PapC usher protein assembles into a ring-shaped complex with a central pore of sufficient size to transport pili across the outer membrane. (A) Blue Native PAGE of purified PapC. Samples were mixed with 0.05% Brilliant Blue G and loaded onto a 5–11% gradient gel. Lane 1 shows the protein standards: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), albumin (67 kDa). Lane 2 shows purified PapC. The slowest-mobility PapC band migrates at a position consistent with a molecular crosslinking, for example, with bis(sulfosuccinimidyl)suberate (BS3, Pierce, Rockford, IL), revealed a ladder of high-molecular-weight crosslinked species. However, these bands were diffuse and difficult to interpret, likely due to intramolecular as well as intermolecular crosslinks. A native gel electrophoresis technique termed “Blue Native PAGE” (51) has yielded the best results for the stoichiometry of the PapC complex. Blue Native PAGE allows membrane proteins to be electrophoresed in the absence of detergent or crosslinker by using Coomassie blue G dye to keep the proteins soluble without denaturation or disruption of protein complexes. The dye also confers a negative charge on the proteins, which will migrate through the gel in response to an electric field and fractionate according to size. Examination of the PapC usher by Blue Native PAGE revealed that the PapC oligomer ran as a series of high-molecular-weight species (Fig. 5) (15). The band of slowest mobility corresponded to a molecular mass of ~550 kDa, consistent with a hexameric complex. The smaller-molecular-weight bands likely reflect dissociation states of the complex, as purification from the outer membrane partially destabilizes the PapC oligomer (15). The oligomeric complexes formed by the secretin family of outer membrane proteins are much more stable than the ushers, resistant even to boiling in SDS (24, 52). The secretins are thought to consist of 12–14 subunits, as estimated by techniques including low-percentage SDS–PAGE and scanning transmission electron microscopy (STEM) (24, 53, 54). STEM allows weight determination by comparison of a protein of interest with a standard of known molecular weight, usually tobacco mosaic virus.

**Blue Native PAGE**

Best results for resolving PapC by Blue Native PAGE were obtained by pouring a large format gradient gel (5–11% acrylamide). Samples were prepared by adding Coomassie brilliant blue G to 0.05% and glycerol (so that the samples sink into the wells) to 5%. These were added directly to PapC in its final storage buffer. The samples were loaded into the gel (without...
any heat treatment) and run overnight at 4°C and 100 V, with 0.01% brilliant blue G in the cathode buffer. The following buffers were used: cathode buffer, 50 mM tricine + 15 mM bis Tris + 0.01% brilliant blue G; gel buffer and anode buffer, 50 mM bis Tris–HCl (pH 7.0). By the end of the run, the gel turns completely blue and visualization of protein bands requires fixing (50% methanol + 10% acetic acid, 45 min), staining (0.025% brilliant blue G + 10% acetic acid, 1–2 h), and destaining (10% acetic acid, 2 h). Protein standards should be run alongside for comparison. The High Molecular Weight Calibration Kit from Pharmacia (Piscataway, NJ) works well (Fig. 5). The standards should be prepared by dissolving in buffer without heating and adding brilliant blue G to 0.05% before loading on the gel. Blue Native PAGE appears to provide a reliable estimate of protein size, but caution must be used when comparing mobilities of native proteins as factors other than size, such as protein shape, can affect migration in the gel.

The usher protein must interact with chaperone-subunit complexes in the periplasm, facilitate dissociation of the chaperone, and translocate the pilus across the outer membrane to the cell surface. The two-dimensional topology of a membrane protein such as the usher can greatly aid in discerning its mechanism of action by revealing which regions of the protein span the membrane and which are exposed on either side of the membrane. Outer membrane proteins appear to span the membrane using β strands arranged into a β barrel instead of the α helices more common for typical membrane proteins (55). β-Barrel outer membrane proteins, such as the porins, are stable to SDS unless heated and exhibit a characteristic temperature-dependent mobility shifting on SDS–PAGE (56, 57). PapC and FimD exhibited this characteristic and thus are likely to also cross the outer membrane as β barrels (15). Computer programs specific for β-strand outer membrane proteins are available for predicting the topology of these proteins (58). Modeling of PapC indicates that it has 24 or more potential membrane-spanning strands, with small extracellular loops and larger periplasmic regions (C. Stathopoulos, D.G.T., R. Curtiss III, and S.J.H., unpublished data). This is consistent with modeling done on the K88 pilus usher FaeD and 987P usher FasD (59, 60). Computer predictions often contain numerous errors, and biochemical techniques such as protease and epitope mapping must be done to confirm predicted transmembrane strands and external and internal loops (61). Bacterial cytoplasmic membrane proteins are often mapped using β-galactosidase and alkaline phosphatase fusions (62), but care must be taken when using large insertions such as alkaline phosphatase to map outer membrane proteins (61). Alkaline phosphatase folds in the periplasm and most likely cannot be translocated across the outer membrane. Therefore, fusions to extracellular loops may give false readings as being in the periplasm. Furthermore, the β-strand structure of outer membrane proteins may not be able to tolerate insertions as well as the α-helical structure of cytoplasmic membrane proteins, leading to alteration or disruption of the protein's structure.

A number of techniques are available for probing usher function. The channel-forming ability of the PapC and FimD ushers was confirmed using a “liposome swelling assay” that has been used extensively for characterization of the bacterial outer membrane pore-forming proteins called porins (15, 63). For swelling assays, a protein of interest is reconstituted into multilamellar liposomes which are then diluted into isoosmotic solutions containing various-sized solutes, usually sugars. Pore-forming ability is detected if the reconstituted protein contains a pore large enough to allow entry of the solute, which will enter the liposomes down its concentration gradient. Water will enter along with the solute which causes the liposomes to swell, resulting in a characteristic decrease in their optical density. The initial rate of this decrease is measured as the pore-forming or swelling activity. By comparing different-sized solutes one can obtain an estimate of pore size. Both the PapC and FimD ushers showed pore-forming activity in swelling assays. The size of the PapC pore was estimated to be on the order of 2 nm in diameter, which agreed well with the electron microscopy results (see above). Great care must be taken, and a number of controls done, when performing liposome swelling assays to avoid artificial results (63).

In addition to serving as a translocation channel across the outer membrane, the usher must also interact with chaperone-subunit complexes in the periplasm and facilitate ordered pilus assembly. Interaction of the usher with chaperone-subunit complexes has been probed using ELISA and overlay (“far Western”) techniques (14). In these assays, the usher was immobilized to a microtiter plate, or subjected to SDS–PAGE and transferred to a membrane. Various chaperone-subunit complexes were then incubated with the usher and binding to the usher determined by probing with antibodies that recognize the chaperone and/or the subunit. These methods revealed that complexes corresponding to the pilus tip bind to the usher, whereas complexes corresponding to the rod bind only weakly or not at all, implying that tips must be made before rods (14). In addition, the chaperone–adhesion complex had the highest affinity for the usher, consistent with its location as the first subunit assembled into the pilus tip. More recently, surface plasmon resonance was used to probe the kinetics of chaperone-subunit–usher interactions for both P and type 1 pili (40). Surface plasmon resonance allows real-time ob-
ervation of association and dissociation events. This analysis was performed using a Biacore 2000 (Pharmacia Biosensor, Piscataway, NJ) instrument. The usher was covalently linked to a biosensor chip and various concentrations of chaperone-subunit complexes were injected over the immobilized usher. Binding of a complex to the usher results in a change in the refractive index of the solution near the chip surface that is detected as a change in the angle of light reflected off the chip. Biacore analysis confirmed results from the ELISA and overlay assays and revealed the basis for the high affinity of the chaperone-adhesin complex for the usher. Chaperone-adhesin complexes from both the P and type 1 pilus systems were found to bind with an association rate an order of magnitude higher than that of the other subunit complexes. Dissociation rates for all of the chaperone-subunit complexes from the usher were found to be slow, arguing that after association of a complex with the usher, the subunit remains bound at the usher ready for interaction with the next incoming complex. This suggests that kinetic partitioning of chaperone-adhesin complexes to the usher is a defining factor in the tip localization of the adhesin.

The precise sequence of events following interaction of chaperone-subunit complexes with the usher that lead to formation of the pilus structure is not known. For example, at what point does the chaperone uncap from the usher? Complicated questions such as these are being probed using in vivo and in vitro techniques. In the type 1 pilus system, pilus assembly intermediates formed in vivo can be copurified along with the usher (40). For example, if the chaperone-adhesin complex is expressed along with the usher and then a standard usher purification performed (using the His tag on the usher), a stable usher-chaperone-adhesin complex can be isolated. Recent data show that even whole pili can be copurified with the usher (E. T. Saulino and S.J.H., unpublished data). Binding of the chaperone-adhesin complex to the usher appears to result in a conformational change in the usher as evidenced by a change in the protease susceptibility of the usher (40). This conformational change was maintained during pilus assembly, suggesting that interaction of the chaperone-adhesin complex with the usher converts the usher to an assembly-competent state. This could indicate a gating role for the chaperone-adhesin complex, perhaps triggering the usher to switch from a "closed" to an "open" state. In fact, production of type 1 pili in several clinical strains is dependent on expression of the adhesin (64, 65).

Reconstitution of pilus biogenesis in a cell-free system using purified components would provide a defined environment for investigation of usher-chaperone-subunit interactions. Cell-free systems have proved very useful for examining the bacterial general secretory pathway and protein translocation into the endoplastic reticulum in eukaryotes (66, 67). A cell-free system has been established for the bacterial outer membrane protein FhuA, which functions in ferrichrome transport and as a bacteriophage receptor, by reconstitution of FhuA into unilamellar liposomes (68). A similar cell-free system for reconstitution of pilus biogenesis may be possible by incorporation of the usher into unilamellar liposomes. Establishment of such a system would allow step-by-step analysis of pilus biogenesis as well as an excellent testing ground for mutants.

**INTERACTION OF PILI WITH HOSTS AND SIGNALING**

Adhesive surface organelles are built for interaction with host cells. Adhesion of bacteria to hosts can initiate signaling events leading to invasion of the bacteria into the host, blockage of uptake into phagocytic cells, or the formation of a specialized niche on the surface of the host such as pedestal formation by enteropathogenic E. coli (EPEC) (69). These signaling events often require transfer of bacterial factors into the host, for example, injection of Yop proteins by the type III secretion system of Yersinia or transfer by EPEC of its own receptor protein into the host (70, 71). Pili have generally been thought to play a passive role in these events, needed only for initial contact with the host. Recent work suggests this may not be correct. Binding of type 1- and P-piliated bacteria to mucosal cells has been shown to initiate host signaling pathways leading to stimulation of cytokine secretion (72). Binding by the FimH adhesin of type 1 pili triggers oxidative bursts from neutrophils and FimH has been demonstrated to activate mast cells and subsequent neutrophil influx (73, 74). Type 4 pili of pathogenic Neisseria appear to stimulate calcium fluxes on binding to epithelial cells (75). Calcium is an important second messenger that impacts a number of cellular response pathways. Pili may also play a role in bacterial invasion into host cells. Binding of FimH-expressing bacteria to macrophages appears to direct internalization of the bacteria by a preferential route, allowing intracellular survival (76). Recent experiments with type 1-piliated bacteria suggest that binding of the FimH pilus adhesin to bladder epithelial cells triggers host signaling pathways, resulting in cytoskeletal rearrangements and possible invasion by the bacteria (77). Identification of the receptor on the host is key for understanding subsequent signaling pathways. Most strategies for identifying the host receptor involve labeling surface-exposed epitopes of the host cell with a tag such as biotin. The host proteins are then solubilized and either incubated with bacteria expressing the
adhesin or passed over affinity columns made up of the adhesin coupled to beads (78, 79). Biotinylated host proteins bound to the adhesin are then identified by running SDS–PAGE, transferring to a membrane, and probing with avidin. Crosslinking strategies and overlay assays can also be used to identify host receptors (80, 81).

Binding of a pilus to its host can also send signals back to the bacteria. Zhang and Normark used a differential display technique to show that binding of P-piliated bacteria to receptor resulted in induction of a number of RNA transcripts, one of which encoded a factor required for iron-starvation response (82). Iron uptake systems are crucial for bacterial growth in low-iron environments such as urine. How does binding to the receptor by the pilus lead to alterations in gene expression in the bacteria? Recent work with the P and type 1 pilus systems suggests a possible model. These pili are assembled from their base (Fig. 2). This assembly is thought to occur at the periplasmic face of the usher, where the chaperone–subunit complexes are converted to subunit–subunit complexes, creating pilus fibers that translocate through the usher to the cell surface. Binding of a pilus to its host might block pilus translocation through the usher, or even cause pilus retraction back through the usher. Pilus retraction would presumably require unraveling of the pilus rod (Fig. 2). Binding of a pilus to its receptor might place mechanical stress on the pilus, which has been determined in vitro to result in unraveling of the rod (3). Consistent with this hypothesis, quick-freeze deep-etch electron micrographs of type 1-piliated bacteria bound to bladder epithelial cells revealed that the bound pili are all extremely short (77). Pilus retraction or blockage of pilus growth would result in a buildup of chaperone–subunit complexes in the periplasm and an increase in off-pathway subunit interactions. Stresses in the periplasm are sensed by at least two signal transduction systems: the CpxA–CpxR two-component regulatory system (83). Both of these pathways activate degP transcription as well as a number of other periplasmic proteins, and both were found to be activated by off-pathway interactions of P pilus subunits (Fig. 2) (9). New studies have shown that the Cpx pathway also affects regulation of the pap operon and may be involved in regulation of other virulence loci such as hemolysin secretion (hly) and cytotoxic necrotizing factor (cnf) (84). Thus, by monitoring pilus assembly status, these periplasmic signaling pathways may control expression of virulence genes that need to be upregulated on contact with the target host cell specified by the pilus adhesin.

CONCLUSIONS

Organelle biogenesis is a complex process. Pilus assembly by the chaperone/usher pathway represents a simple and elegant solution to this problem, requiring only two specialized assembly components. Other bacterial secretion and assembly systems, such as type 1 pilus biogenesis and the type II and III secretion systems, can require more than 20 assembly components. Thus, the chaperone–usher pathway, by virtue of its simplicity, provides an excellent model for furthering our understanding of secretion and organelle biogenesis. The periplasmic chaperone family, exemplified by PapD, allows study of the action of postsecretory chaperones and chaperones involved in organelle building. The action of the periplasmic chaperone also has parallels with the role of chaperones in the import of newly synthesized proteins into the endoplasmic reticulum. All Gram-negative bacterial secretion systems face the problem of exporting their substrates, which are often already folded, across the outer membrane. As exemplified by the usher and secretin families, a common solution is the use of integral membrane proteins that assemble into large ring-shaped complexes with central pores. The usher provides a model not only for understanding how proteins cross the bacterial outer membrane but also for understanding how the assembly of complex structures is controlled and ordered.

A multidisciplinary approach has been the key to the successes in picking apart the molecular mechanisms underlying pilus assembly by the chaperone/usher pathway. Future developments in pilus biogenesis and in organelle biogenesis in general will require more of this same approach. Pili are virulence organelles. Therefore, in addition to furthering our knowledge of complex assembly processes, a greater understanding of pilus biogenesis will create new opportunities for the development of novel antimicrobial agents and vaccines.

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