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STRUCTURE-FUNCTION AND BIOGENESIS OF THE TYPE IV PILI

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ABSTRACT

Type IV pili are adhesins expressed by a number of diverse gram-negative microorganisms. These pili are related through similarities in the primary amino acid sequences of the structural subunits, a conserved assembly machinery, and a similar mechanism of transcriptional regulation. Type IV pilus assembly is preceded by proteolytic processing and *N*-methylation of the pilin polypeptide. This process is carried out by a novel bifunctional enzyme PilD, first identified in *Pseudomonas aeruginosa*. Moreover, proteins homologous with type IV pilins have been shown to function in extracellular protein secretion in gram-negative bacteria and in transformation competence in gram-positive microorganisms. Like prepilin, these proteins are also processed and *N*-methylated by PilD. Transcription of the genes for type IV pilins is carried out by an RNA polymerase with a minor sigma factor, RpoN. In *P. aeruginosa* two other regulatory elements (PilS and PilR) are required for pilin expression. RpoN, but not PilS and PilR, is required for expression of a diverse set of bacterial genes. Therefore, regulation of synthesis and posttranslational modification and assembly of type IV pili serves as a useful model for a number of diverse biological processes in the bacterial cell.

INTRODUCTION

Many bacteria display surface appendages called pili (fimbriae) that function by mediating interaction of bacteria with surfaces of other cells. Conjugal DNA transfer is preceded by recognition of the recipient cell by the pili of the donor cell, whereas attachment of pathogenic bacteria to membranes of eukaryotic cells is mediated by the lectin component of pili. In addition to these adhesive functions, pili have been implicated in a mechanism of bacterial locomotion, called twitching motility.

Investigators have identified distinct families of pili by determining the specificity of host-receptor recognition and the seroreactivity of antibodies against pilin proteins and by comparing deduced amino acid sequences of cloned pilin structural genes. The resulting pili families in some cases include pili from taxonomically dissimilar bacteria.

This review attempts to summarize the current knowledge of pili collectively designated type IV pili. Several bacterial species produce these structures, and although they have not been implicated in conjugal DNA transfer, they are important virulence factors because of their role as bacterial adhesins. Moreover, recent work has revealed that proteins with extensive sequence similarity with the type IV pilins are essential components of biological processes such as extracellular protein secretion from gram-negative bacteria and DNA uptake by gram-positive microorganisms. Two important

properties associated with expression of type IV pili, antigenic and phase variation, are not discussed here because they have been subject of several recent reviews (66, 94).

CHARACTERISTICS OF THE TYPE IV PILI FAMILY

Pili are grouped from a rather divergent collection of gram-negative microorganisms to form the type IV class according to similarities in amino acid sequence of the pilin polypeptide. The second conserved characteristic is the occurrence of *N*-methylated amino acids (phenylalanine or methionine) as the first amino acid of the mature pilin structural subunit. Alignment of amino acid sequences of the precursors of the type IV pilins (Figure 1) demonstrates the basis for the assignment of these proteins into a related family. The type IV pilin family can be further divided into two groups. Group A consists of pilins from *Pseudomonas aeruginosa* (40), *Neisseria gonorrhoeae* (65), *N. meningitidis* (82), *Moraxella bovis* (59), and *Dichelobacter* (formerly *Bacteroides*) *nodosus* (63). More recently, this class was expanded to include the pili of *Moraxella lacunata* (58), *Moraxella nonliquefaciens* (116), *Branhamella catarrhalis* (60), and *Eikenella corrodens* (89, 117). The pilins in this group are synthesized as precursors with unique short, basic, amino-terminal leader peptides. These leader sequences are removed by endoproteolytic cleavage between an invariant glycine residue and a phenylalanine residue prior to assembly of the pilin monomers into pili (Figure 1). The amino acid sequence homology of this family extends into the mature polypeptide and is most pronounced near the amino terminus. Because the amino-terminal domain of these polypeptides is highly conserved, it is often referred to as the constant domain. This domain has a strong hydrophobic character interrupted by an invariant glutamic acid residue located exactly five amino acids from the mature amino terminus. The middle of the polypeptides are considerably less homologous and contain the variable domains that comprise the antigenic epitopes of these pili. Another region of homology near the carboxy terminus contains a characteristic pair of cysteines forming a disulfide loop.

The second group of the type IV pilin family, group B, currently has two members, the subunits of the toxin-coregulated pili (TCP) of *Vibrio cholerae* (26, 95), and the subunits of bundle-forming pili (BFP) (19) of enteropathogenic *Escherichia coli*. These proteins are homologous with each other and with the group A members near their amino termini, including the location of the invariant glutamic acid within the hydrophobic region. Although the carboxy-terminal regions of both TcpA (the TCP subunit) and BfpA (the BFP subunit) differ, they do contain a pair of cysteines that presumably form an intrachain disulfide bond.

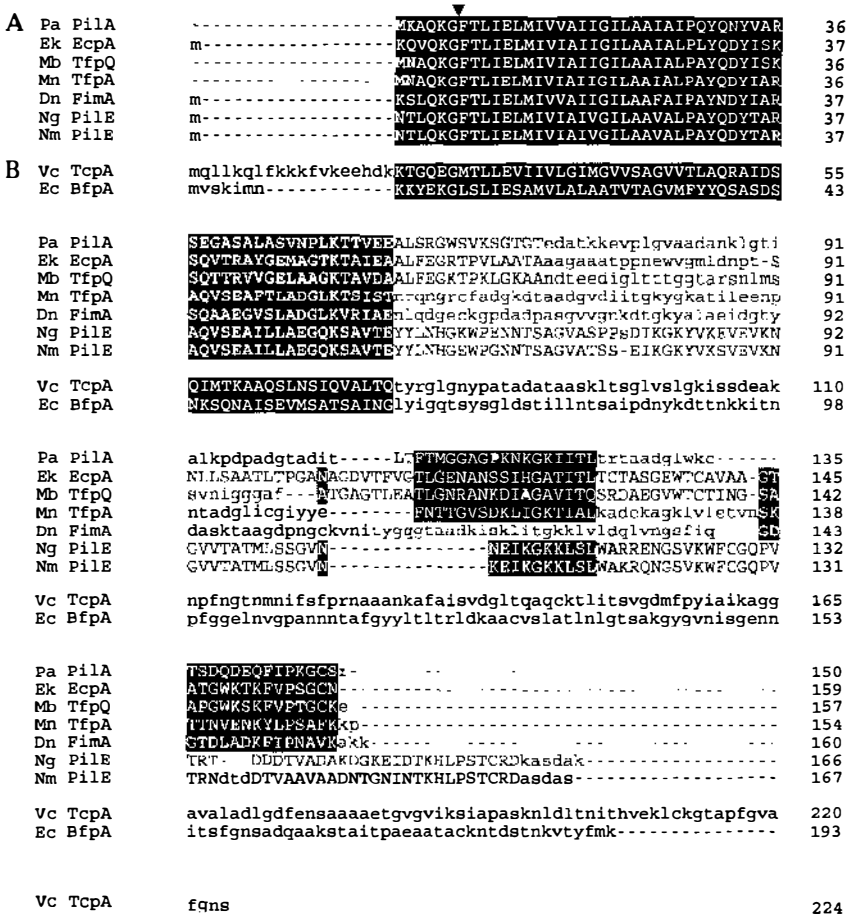

Figure 1 Comparison of the amino acid sequences of representative group A and group B type IV pilins. Species abbreviations with the structural subunit designation are: Pa, *P. aeruginosa,* strain PAK (41); Ek, *E. corrodens,* strain ATCC 23834 (89); Mb, *M. bovis,* strain EPP63 (Q pilin) (28); Mn, *M. nonliquefaciens,* strain NCTC 7784 (116); Dn, *D. nodosus,* strain AC6 (7); Ng, *N. gonorrhoeae,* strain MS11 (6); Nm, *N. meningitidis,* strain C311 (82); Vc, *Vibrio cholerae,* strain Z17561, classical (26); Ec, *Escherichia coli,* E2348/69 (19). This alignment was done using MACAW (Multiple Alignment Construction and Analysis Workbench from the National Center for Biotechnology Information), which searches for and aligns regions of functional similarity using a strategy of pairwise comparison of multiple sequences (93). The shaded regions with the single-letter amino acid code in capital letters indicates the regions of highest similarity. White lettering on black indicates the presence of a particular block of similarity in more than 67% of the input sequences, white on gray between 37 and 66%, and black capital letters on white representing a homologous block found in at least two of the sequences. The cleavage site of the type IV pilins is denoted by an upside-down triangle.

The precursors of TcpA and BfpA are synthesized with longer leader peptides than those of group A: 25 and 13 amino acids, respectively, with a net basic character. The signal peptides of TcpA and BfpA end with glycine, as is the case with group A members. The first amino acid of mature TcpA is methionine, and that of BfpA is leucine, which contrasts with the presence of invariant phenylalanine found at the mature amino termini of group A members. The amino-terminal methionine of mature TcpA is *N*-methylated, but the modification of the amino-terminal leucine of BfpA has not been determined. The conservation of sequence near the amino terminus surrounding the cleavage site places these proteins into the type IV pilin family, and a further conservation of the machinery involved in biogenesis is expected. This machinery includes the leader peptidase and *N*-methyltransferase for removal of the leader peptide and methylation of the amino terminus, a common mechanism of secretion across the membrane and possibly a conserved mechanism for assembly of subunits into mature pili.

STRUCTURE OF THE TYPE IV PILUS ORGANELLE

The pili belonging to group A are flexible, approximately 6 nm in diameter, and have a length ranging from 1000 to 2500 nm. Each type IV pilus is estimated to contain 500–1000 subunits, based on their average length and assuming that they are indeed composed of a polymer of a single polypeptide. The pilin subunits of *P. aeruginosa* appear to be arranged in a helical manner, with approximately five subunits per turn around the long axis of the filament (76). More recently, the crystallization of *N. gonorrhoeae* pilin and subsequent X-ray diffraction analysis (77) has led to the proposal that each individual pilin subunit folds into an antiparallel 4-α-helix bundle similar to tobacco mosaic virus coat protein and myohemerythrin (29, 78). Dissociation of pili from either *P. aeruginosa* or *N. gonorrhoeae* with nonionic detergents yielded a stable dimeric form of the pilin subunit that appears to be the initial building block leading to the polymerized helical structure. The assembly therefore involves interaction of subunits to form dimers, followed by assembly of higher-order structures from the dimerized subunits. However, dimerized subunits have been isolated only from assembled pili, and dimerized pilin has not been shown to be an obligatory precursor of organelle assembly. A detailed biochemical analysis of *P. aeruginosa* pilin, involving alkaline pH titrations, solvent perturbation, quenching of tryptophan fluorescence with acrylamide, and circular dichroism, demonstrated that tyrosine residues at positions 24 and 27 in the hydrophobic domain are at a dimer/dimer interface in both native pili and in reassembled pilin filaments (122, 123). Dissociation of pili by octyl glucoside resulted in exposure of these tyrosines. These two

residues are conserved in all type IV pilins of group A , but are not present in TcpA or BfpA.

The type IV pilins of group B (TcpA and BfpA) assemble into straight fibers approximately 7 nm wide and of variable length (30, 114). Little structural information is available on the arrangement of the subunits within the filament. One of the striking features of these pili is their propensity to aggregate laterally when expressed on the bacterial surface or when purified. Conceivably, aggregation of filaments between individual bacteria may allow for formation of microcolonies in their natural environment or in infected tissues.

ROLE OF TYPE IV PILI IN VIRULENCE

Adherence to Epithelial Cells

Type IV pili have been found on gram-negative pathogens that cause a variety of diseases in animals and humans. The contribution of pili to virulence lies primarily in their ability to promote attachment to various types of receptors during tissue colonization. In contrast, bacterial attachment to phagocytic cells, mediated by pili or through antipilin opsonic antibody, is an important host-defense mechanism. Evidence for the role of pili in pathogenesis is examined here for selected microorganisms.

P. AERUGINOSA *P. aeruginosa* possesses several adhesins that allow binding to a variety of epithelial cell types and mucins, which are amino sugars found in the secretions that bathe respiratory-tract cells. *P. aeruginosa* pili form one class of these adhesins and have been extensively studied from this perspective. Initial observations by Woods et al (126), who first suggested a role for pili in bacterial adherence, have been confirmed in several tissue culture systems. While the contribution of pili to virulence of *P. aeruginosa* has not been extensively investigated, piliated organisms were 10-fold more virulent than nonpiliated strains in a burned mouse model. The susceptibility of healthy mice to different strains did not vary regardless of the strain's piliation (92).

Pili can function in bacterial adhesion by direct interactions of the assembled pilin subunit with a tissue receptor. Alternatively, pili can incorporate minor pilin-like subunits that are responsible for receptor recognition and binding. Cumulative evidence suggests that pilin subunits do recognize certain cellular receptors. Many bacteria expressing type IV pili, however, do express other adhesins on their surface (79, 97).

Two approaches demonstrated the involvement of pili in bacterial adhesion. Monoclonal antibodies specific for defined regions of the pilin monomer,

purified pili, and synthetic peptides corresponding to specific peptide sequences within the pilin subunit inhibited bacterial attachment to buccal and tracheal epithelial cells (16–18, 37, 52, 53). Moreover, peptides containing the cysteine-cysteine bridge region were more efficient in blocking bacterial adherence than peptides specific to other epitopes recognized by monoclonal antibodies. Also, antisera raised against oxidized peptides containing the cysteine residues inhibited adherence of both homologous and heterologous strains (37, 53). These studies suggested that the binding domain on the pilus for epithelial cells contained the cysteine-cysteine bridge, and that this region was also a conserved antigenic determinant.

Evidence from several laboratories has raised the possibility that additional adhesins, not associated with pili, function as *P. aeruginosa* adhesins. Loss of functional pili reduces adherence to cultured epithelial cells (13, 97) and bovine tracheal cells (91), but does not abolish it, while adhesion of *P. aeruginosa* to mucins, or carbohydrate components of mucin, is not affected by mutations in the pilin structural gene (87, 88). Interestingly, certain regulatory mutations that abolish expression of pili result in complete loss of bacterial adherence to most cells and to mucin (13, 87). This suggests the possibility that expression of multiple adhesins by *P. aeruginosa* is regulated by one or several common regulatory elements.

In addition to mediating adherence to epithelial cells or mucin, pili of *P. aeruginosa* may be the targets recognized by phagocytic cells. Speert and coworkers have demonstrated that the presence of pili on the surface of nonmucoid *P. aeruginosa* was required for nonopsonic phagocytosis by human neutrophils and monocyte-derived macrophages (98, 99). Furthermore, fibronectin-stimulated macrophages phagocytize *P. aeruginosa* cells grown in vivo, on nutrient agar plates, or in static broth (46–48), conditions known to allow bacteria to achieve maximal piliation. The same strains grown in agitated cultures were not taken up, and electron microscope examination showed that surface pili were missing, presumably because of the shearing action of the growth conditions. In addition, a pilin gene transposon mutant also was not phagocytized, leading to the conclusion that pili serve as ligands for fibronectin-stimulated macrophages and subsequent phagocytosis.

N. GONORRHOEAE Pili appear to play an essential role in infections of human hosts by *N. gonorrhoeae,* the organism responsible for the sexually transmitted disease gonorrhea (10). The organism adheres to a variety of cultured epithelial cells and, as with all such adhesins, it is postulated that the gonococcal pili facilitate the initial attachment to and colonization of human mucosal tissues prior to invasion and development of overt disease (108, 120). Evidence for involvement of pili in interaction of pathogen with host cells is based primarily on studies of in vitro attachment using organ and

cell-culture systems. Piliated gonococci mediate adherence to cultured vaginal epithelial cells (57), human fallopian tubes (62), and erythrocytes (11). Monoclonal antibodies against peptides corresponding to constant and variable regions of the pilus prevent adherence to a human endometrial carcinoma cell line (90), suggesting that pilin polypeptides themselves mediate adhesion to cells. However, more recent studies have demonstrated that surface proteins other than pili mediate adherence of *N. gonorrhoeae* to specific glycolipids, such as lactosylceramide and gangliotriaosylceramide derived from a human cervical carcinoma (14, 107). A nonpilus adhesin has been isolated and cloned and has been shown to be a 36-kDa protein on the surface of gonococci that is not associated with pili (79).

DICHELOBACTER NODOSUS *D. nodosus* is responsible for ovine footrot, a contagious, debilitating disease of sheep (24). The common type IV pilus of *D. nodosus* has been established as the major host-protective immunogen (101), and vaccines prepared either from purified pili or piliated whole cells offer protection against homologous challenge (100). In fact, this is the only vaccine generated against a type IV pilin that has proven effective in prevention of disease. There are no reports addressing the role of pili in adherence. Moreover, it is not clear whether the efficacy of the pilus-based vaccine results from its ability to interfere with adherence or from increased opsonophagocytosis of the bacteria.

MORAXELLA BOVIS *M. bovis* is the causative agent of infectious bovine keratoconjunctivitis, the most common ocular disease of cattle worldwide. Pili are considered to be a virulence factor in the disease because only piliated organisms cause infections by mediating attachment (2, 12, 39, 68), under experimental conditions (55). Vaccination of cattle with pili of *M. bovis* has been shown to be protective against homologous challenge (54).

NEISSERIA MENINGITIDIS *N. meningitidis* is a causative agent of bacterial meningitis and sepsis, especially in children. Pili expressed by this pathogen fall into two distinct classes. Class I pili consist of pilin subunits that share serological and amino acid–sequence similarity to the members of the type IV family, whereas class II pili appear to be unique (82, 119). Interaction of piliated organisms bearing class I or class II pili with a variety of cultured cells was investigated (118). Class I pili were the primary determinant of binding to three different human epithelial cell lines and an endothelial cell line. To further investigate the correlation between piliation and adherence, Nassif et al (69) isolated variants of a nonpiliated *N. meningitidis* that express class I pili but differ in their ability to bind to human endometrial carcinoma cells. All adhering piliated meningococci expressed one antigenic variant

while the nonadhering variants expressed another. Transformation of nonadhering meningococci with the pilin gene from adhering variants conferred adherence on the recipient. These data suggest that only certain pilin serotypes can function as adhesins.

PILI OF *V. CHOLERAE* AND ENTEROPATHOGENIC *E. COLI* Colonization of the small intestine by *V. cholerae* and subsequent production of toxins causes the clinical symptoms of cholera. The disease is characterized by a profuse diarrhea caused primarily by electrolyte imbalance following the actions of the cholera enterotoxin on intestinal epithelial cells. Because of the peristaltic action of the intestinal tract, attachment of the pathogen to the intestinal lining is an important event during the host-pathogen interaction. The direct involvement of *V. cholerae* TCP in the pathogenesis of cholera was demonstrated in an infant mouse model, in which a dramatic increase in the infectious dose occurred with a strain bearing a mutation in TcpA, the pilus structural subunit (114). The same mutant was unable to colonize the mouse intestinal tract, establishing the role of TCP as a colonization factor. The function of TCP as a potential adhesin was confirmed by demonstrating the inability of *tcpA* mutants to agglutinate erythrocytes. The precise nature of the receptor for TCP is not known.

Enteropathogenic *E. coli* (EPEC) are important agents of diarrheal disease of children in developing countries. The EPEC isolates can adhere to a variety of tissue-culture cells in a pattern called localized adherence, in which bacteria form clusters on the epithelial cell surface. The bundle-forming pili mediate the localized adherence of EPEC because this action can be blocked by antibodies to BFP (30). Moreover, EPEC strains carrying transposon insertions in *bfpA* have lost the ability to adhere to epithelial cells. Purified BfpA can mediate hemagglutination of human or mouse red blood cells. Although BFP and TCP have many structural similarities, there is no evidence that the receptors for the pili of these intestinal pathogens are similar.

BIOGENESIS OF TYPE IV PILI

Pilus biogenesis includes the concerted synthesis of major and minor subunits, various posttranslational modifications, and assembly into a functional organelle. This process likely involves one or more accessory proteins. Genes responsible for biogenesis of *E. coli* pili are often clustered near the structural subunit genes, presumably to allow for coordinate transcriptional control (36, 50, 75). Type IV pilin genes are all located in the bacterial chromosome, with the exception of the BfpA pilin gene of enteropathogenic *E. coli*, which is located on the 92-kilobase virulence plasmid (30). However, the structural subunit genes are usually surrounded by genes that do not encode biogenesis

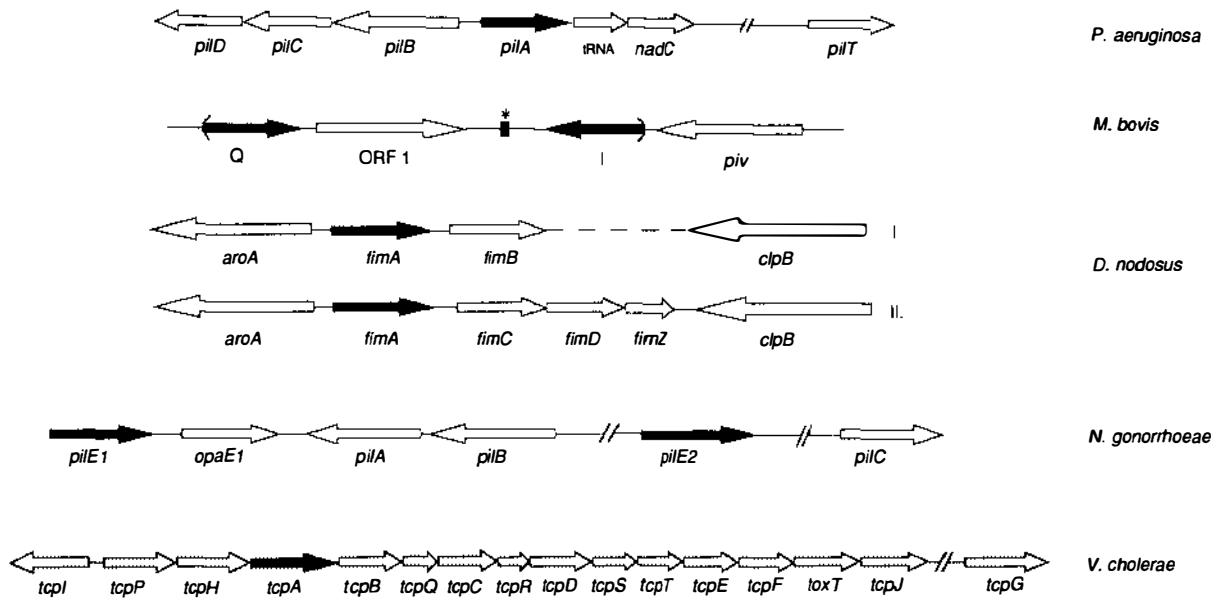

Figure 2 Summary of genetic organization of type IV pilin/fimbrial structural and biogenesis genes. The figure shows the level of diversity in gene organization around each type IV pilin gene. The gene encoding the major pilin/fimbrial subunit is shown as a black arrow, with the genes immediately adjacent shown as white arrows. Touching arrows depict genes transcribed as an operon, whereas genes with individual transcriptional start sites are arrows separated with lines. The brackets on the *M. bovis* map indicate the location of the inversion region leading to expression of either Q or I pilin (the orientation show is for Q expression); the asterisk indicates the position of the *hin*-like recombinational enhancer sequence. The gene *piv* is the putative invertase in this system. The two-expression loci version of *N. gonorrhoeae* MS11 is shown, although many strains only have one expression locus; the silent pilin loci are not shown here. Separating bars denote important genes in pilus biogenesis that are not clustered with the genes directly around each pilin subunit gene. Genes unrelated to type IV pilus biogenesis include a tRNA gene and *nadC* located downstream of *pilA* in *P. aeruginosa*, *aroA* and *clpB* in *D. nodosus*, and *opaE1* in *N. gonorrhoeae*. In addition, the functions encoded by genes *fimB*, *fimC*, and *fimD* in *D. nodosus* have not been determined. References for genetic organization: *P. aeruginosa* (32, 70, 125; D. N. Nunn, personal communication); *M. bovis* (28, 58); *D. nodosus* (33); *N. gonorrhoeae* (65, 110); *V. cholerae* (74, 112).

functions. As shown in Figure 2, the genes required for *V. cholerae* TCP assembly are the only type IV biogenesis determinants extensively linked with the pilin structural subunit gene. Therefore, TCP most closely resembles the genetic organization of *E. coli* pili, as exemplified by the *pap* pilus gene cluster (36).

Although the region of the chromosome flanking the structural subunit of type IV pili fails to show a conserved biogenesis machinery, the members of this family are doubtless assembled by a similar mechanism. Expression of *D. nodosus, M. bovis,* and *N. gonorrhoeae* pilin genes in *P. aeruginosa* results in the formation of pili in the heterologous host (5, 25, 34, 61). These studies show that the basic machinery involved in biogenesis of the type IV group A pili appears to be conserved. The various posttranslational modifications and the ordered assembly of subunits involves recognition of structural features of the pilin polypeptide found in all members of this group. Similar heterologous expression and assembly of TCP and BFP by those bacteria that express type IV group A pilins has not been reported.

Genetic Determinants of Type IV Pilus Biogenesis in P. aeruginosa

The first isolation of genes directly involved in biogenesis of prototypical type IV pili was accomplished by localized transposon mutagenesis of the chromosomal region flanking the pilin structural gene (*pilA*) by Nunn et al (70). The effects of these chromosomal transposon insertions on the assembly of pili were monitored by electron microscope examination and by observing mutant sensitivity to killing by the pilus-specific bacteriophage PO4. The transposon insertions revealed that a 4.0-kilobase (kb) region adjacent to the pilin structural gene was required for the formation of mature pili. DNA sequencing revealed the presence of three open reading frames designated *pilB, pilC,* and *pilD* that encode 62-, 38-, and 32-kDa proteins, respectively. The predicted sizes of these proteins were confirmed by expression in *E. coli* using a T7 promoter-polymerase expression system. Although mutations in either *pilB, pilC,* or *pilD* resulted in the absence of pili on the bacterial surface, they all synthesized pilin antigen in the cell at a level comparable with that produced by the wild-type bacteria. The functions of PilB and PilC in biogenesis is not clear, but the precise role of PilD has been elucidated.

P. aeruginosa *PilB and PilC*

P. aeruginosa containing mutations in either *pilB* or *pilC* are pilus-deficient but express pilin that is localized predominately in the cytoplasmic membrane (70). The pilin subunits expressed by *pilB* or *pilC* mutants appear to be in the fully processed form (i.e. the six–amino acid leader peptide is removed); hence the defect in these mutants occurs during assembly of pilin following interaction

of the subunits with the membrane. PilB lacks an identifiable secre- tion-signal sequence, and hydrophobicity analysis of the deduced polypeptide sequence suggests that it is a cytoplasmic protein. PilC, however, may be an integral cytoplasmic membrane protein resulting from the presence of four po- tential membrane-spanning domains and absence of a typical signal sequence.

The predicted amino acid sequences of PilB share significant similarity with several other proteins, two of which are expressed by *P. aeruginosa*. One of these is PilT, a protein required for twitching motility in *P. aeruginosa* (125). *P. aeruginosa* XcpR (4) is also similar to PilB, suggesting that these proteins arose by gene duplication from an ancestral gene. This similarity extends to the predicted amino acid sequences of ORF1 from the *Bacillus subtilis comG* operon, to PulE in *Klebsiella oxytoca,* and to a lesser extent to VirB-11 of *Agrobacterium tumefaciens* (125). Although the similarity extends across the entire sequences of these proteins, there are two notable conserved regions. The first conserved domain contains a consensus nucleotide binding se- quence, GXXXXGK(T), which is common to many nucleotide-binding proteins from both prokaryotes and eukaryotes (121). The other prominent homologous region contains the less strongly conserved secondary domain (R/KXXXGXXXL-4 hydrophobic-D), which is present in only some nucle- otide-binding proteins (27, 121). Therefore, PilB (and PilT) are probably cytoplasmic nucleotide-binding proteins. One possible function for PilB is to supply energy for subunit translocation or assembly. Because many of the PilB homologues are involved in movement of macromolecules across biological membranes, they may also provide energy for the translocation processes as well.

P. aeruginosa *PilD Is a Leader Peptidase*

The transposon insertions identified a third gene, *pilD,* located distal from the pilin structural gene (70). The predicted amino acid sequence of PilD showed a protein of high hydrophobicity, with five to six transmembrane- spanning helices and without a typical signal peptide. This suggested the possibility that PilD is an integral cytoplasmic membrane protein, which was confirmed by localization studies using antibodies to PilD.

Studies of *P. aeruginosa pilD* mutants demonstrated a complete loss of pili with an accumulation of precursor, unprocessed prepilin in the bacterial cell envelope. The product of the *pilD* gene, therefore, could represent a leader peptidase or a component essential for the prepilin-processing reaction, catalyzed by another protease. To directly examine the role of PilD in leader-peptide cleavage, PilD was purified to homogeneity by immunoaffinity chromatography using antibodies raised against a peptide deduced from the *pilD*-coding sequence. Purified PilD, in the presence of nonionic detergents and phospholipids, could cleave the leader sequence from purified prepilin as

well as from prepilin of *N. gonorrhoeae*, another member of the type IV group A family (71).

N-*Methyltransferase Activity of PilD*

One of the characteristics of the type IV pilin family is that the first amino acid of the mature protein (Phe or Met) is *N*-methylated following cleavage of the leader peptide. The type IV prepilin peptidase, PilD, in addition to cleaving the six–amino acid leader peptide, is the same enzyme that catalyzes the *N*-methylation of the amino-terminal residue in *P. aeruginosa* pilin (106). The bifunctionality of the enzyme has been clearly demonstrated using purified PilD, purified prepilin, and *S*-adenosyl-L-methionine (Ado-Met) as the methyl donor. In addition, PilD can recognize and efficiently methylate pilin that has been first cleaved in the absence of the methyl donor, thus suggesting that cleavage and methylation are independent reactions, perhaps involving two different active sites. The latter was confirmed by demonstrating that the methyltransferase activity of PilD can be competitively inhibited with Ado-Met analogues without affecting peptidase activity.

Other bacterial prepilin leader peptidases that are homologues of PilD very likely have *N*-methyltransferase activity as well. PulO, a protein similar to PilD that is involved in maturation of components of the extracellular secretion apparatus of pullulanase in *Klebsiella oxytoca* (see below), cleaves the gonococcal prepilin when both are expressed in *E. coli* (22). Moreover, the amino-terminal residue of this mature pilin could not be identified by standard amino-terminal sequencing, very likely because of a coupled modification, leading Dupuy et al (22) to speculate that either *E. coli* has a similar *N*-methyltransferase activity or that PulO is also the *N*-methyltransferase. The bifunctionality of PilD may very well be conserved among all of the type IV peptidases. Using a *P. aeruginosa pilD* probe, Dupuy et al identified a clone that very likely encodes the gene for *N. gonorrhoeae pilD*. Another group has recently sequenced the *N. gonorrhoeae pilD* homologue (51a). A comparison of the deduced amino acid sequence of $PilD^{GC}$ to *P. aeruginosa* PilD demonstrated a 48% identity and 65% similarity between the two proteins. TcpJ, the prepilin leader peptidase of *V. cholerae* (44), may also be a similar bifunctional enzyme, because the methionine of the TcpA structural subunit is completely methylated (95).

Structure-Function of PilD

Hydropathy analysis of PilD shows a typical membrane protein with at least five putative transmembrane regions. A single large, relatively hydrophilic domain contains a region that is most conserved among PilD homologues (TcpJ, PulO, OutO, ComC, and $PilD^{GC}$) (44, 51a, 56, 67, 86). The location of this domain in the cytoplasm was confirmed by fusing alkaline phosphatase

and β-galactosidase to PilD in the middle of this hydrophilic stretch (101a). The former hybrid protein lacked alkaline phosphatase activity, while the latter showed high levels of β-g
cytoplasmic location of this segment of the PilD polypeptide. This region is also notable for the presence of four cysteine residues, arranged in a pairwise fashion with the cysteines of each pair either adjacent or separated by 2 amino acids with approximately 22 amino acids between pairs (44, 51a, 101a, 125).

Examination of the effects of various inhibitors showed that the cysteine-specific alkylating agents, *N*-ethylmaleimide (NEM) and iodoacetamide, as well as reversible agents such as *p*-chloromercuribenzoate, inhibit both the prepilin peptidase and *N*-methyltransferase activities of PilD (101a). The role of cysteines in catalysis was confirmed by site-directed mutagenesis of the codons for all five cysteines in *P. aeruginosa* PilD. Substitutions of individual cysteines with serine or glycine within the conserved hydrophilic domain of PilD substantially reduced both leader peptidase and *N*-methyltransferase activity. Similar substitutions of the single amino-terminal cysteine found within the amino-terminal hydrophobic segment had no effect on either activity. Therefore, the active sites of the enzyme are within adjacent domains of the cytoplasmic segment of PilD. Other parts of PilD, however, could participate in proteolysis and methylation, including segments within the membrane. Interestingly, none of the mutations completely abolished the activity of the enzyme, and even substitutions that lowered the enzymatic activity of PilD to 5% of the wild-type allowed bacteria to assemble sufficient pili to make the mutant bacteria susceptible to killing by the pilus-specific phage PO4.

The Specificity and Kinetics of Leader-Peptide Cleavage and N-*Methylation of Mature Pilin*

The cleavage site for the type IV pilin precursors is between glycine and phenylalanine residues (methionine in TcpA) in the sequence Gly-Phe(Met)-Thr-Leu-Ile(Leu)-Glu, which is conserved among all members of the type IV pilin family (Figure 1). The precise role of the leader sequence is not known. Although the sequence cannot function as an export signal, membrane insertion of pilin is very likely directed by the hydrophobic portion of the mature polypeptide, based on the cellular localizations of pilin-alkaline phosphatase (PhoA) fusions (102).

Attempts to define the minimal sequence of prepilin that still functions during the initial stages of pilus biogenesis relied on engineering of gene fusions between the deleted pilin gene from *P. aeruginosa* and the *E. coli phoA* gene. The hybrid proteins expressed from the gene fusions contained the entire six–amino acid leader sequence, along with 3, 26, 41, and 46 residues of the mature protein fused to PhoA at the carboxy terminus. The hybrid proteins containing the 26-, 41-, and 46–amino acid portion of the

pilin were translocated across the cytoplasmic membrane, as indicated by the high levels of PhoA activity in *P. aeruginosa* (102; M. S. Strom, unpublished results). It was not possible to determine leader-sequence cleavage of the PilA-PhoA fusions. However, when purified, only the largest hybrid was efficiently methylated by PilD, suggesting that the 46–amino acid amino-terminal portion of pilin, together with the leader sequence, contains the necessary information for translocation across the cytoplasmic membrane, with concomitant leader-peptide cleavage and *N*-methylation. The smallest fusion did not direct translocation of PhoA across the inner membrane, and neither it nor the hybrids containing 26 and 41 amino acid residues of mature pilin were *N*-methylated in vitro in the presence of PilD and Ado-Met (102; M. S. Strom, unpublished results). Similarly engineered hybrid proteins between *N. gonorrhoeae* pilin and PhoA that contained a 20- or 34–amino acid segment of the gonococcal prepilin were also not processed in vivo (23).

Oligonucleotide-directed site-specific mutagenesis of *pilA* was used to construct a series of amino acid substitutions in the pilin precursor to identify the specific amino acids within the amino terminus of prepilin of *P. aeruginosa* that are important for processing, posttranslational modification, and assembly of the pilin subunits into mature pili (103). This mutational analysis showed that the glycine residue at the −1 position relative to the leader peptide cleavage site is absolutely required for prepilin processing prior to pilus biogenesis. Substitution of alanine for glycine at this position resulted in partial processing of prepilin, suggesting that an amino acid with a small side group was required at this position for cleavage by PilD.

A second class of mutations engineered in the pilin protein of *P. aeruginosa* centered around the phenylalanine at the +1 position. This residue can be substituted by a variety of other residues with little effect on formation of pilin, as assessed by electron microscopy and sensitivity to killing by the pilus-specific phage PO4. Substitutions within the leader peptide that increased the net charge to +3 from +2, or lowered it to +1, 0, or −1, had no effect on either pilin processing or pilus biogenesis. However, the leader sequence itself is necessary, because a deletion mutation that removed this sequence resulted in unstable pilin expression and no pilus assembly.

Additional mutations in the mature portion of *P. aeruginosa* prepilin were also examined for processing and pilus assembly (80). Prepilin with a deletion of eight residues (from Ile+4 to Ala+11 of the mature protein) resulted in a form that was not proteolytically processed, whereas a four–amino acid deletion (from Ile+4 to Met+7) was partially processed. The construct with the four–amino acid deletion, but not the eight, was still incorporated into the membrane. Apparently, these deletions altered the overall hydrophobic character of the mature portion, which then affected recognition of the prepilin

substrate by PilD. Interestingly, the majority of point mutations in the hydrophobic domain near the amino terminus of pilin have little or no effect on processing of prepilin or its assembly into pili (103). The only exception is an invariant glutamic acid, found at the +5 position from the cleavage site. Substitutions at this position with either hydrophobic or basic amino acids have no effect on prepilin processing, but the pilin is not *N*-methylated and subunits are not assembled into pili (80, 103). Furthermore, the same mutant pilin was not a substrate for *N*-methylation in vitro by PilD (M. S. Strom, unpublished results). Although the presence of an acidic residue within the hydrophobic amino-terminal domain is not essential for cleavage of the leader sequence by PilD, it may be part of the recognition sequence for the methyltransferase activity catalyzed by the same bifunctional enzyme.

To assess whether phenylalanine at the amino terminus is the only residue that can be *N*-methylated, several mutations at the +1 position were examined for the presence of *N*-methylated amino acids (103). Substitutions of methionine, alanine, and tyrosine resulted in processed pilin with the substitute residue almost fully *N*-methylated. However, two mutations gave opposing results. In the first, a serine residue at this position was not methylated although pili were produced. In the second, a glycine substitution resulted in fully processed pilin that was approximately 50% *N*-methylglycine at the amino terminus, but no functional pili were detected by phage PO4 sensitivity or by electron microscopy. These results suggest that PilD, which catalyzes the *N*-methylation of pilin, can modify a wide range of amino acids. Although in some mutants no obvious defects result from lack of methylation, the pili assembled from unmethylated subunits may differ in some subtle way from methylated pili. This difference would not be obvious when piliated bacteria are examined by electron microscopy.

The kinetics of cleavage of the leader peptide from the *P. aeruginosa* pilin precursor was also determined (104). A K_m of approximately 650 μM and a turnover rate, or K_{cat}, of 180 min^{-1} were measured. Similar rates were obtained when PilD-mediated processing of the *N. gonorrhoeae* prepilin was measured. Therefore, the differences in length and net charge of the *N. gonorrhoeae* prepilin leader sequence does not affect the overall rate of cleavage by PilD. The rates of processing of prepilins obtained by site-directed mutagenesis of the codon for the phenylalanine at the +1 position were lower than those of the wild-type substrate. Substitution of a methionine at this position resulted in substrate that was processed at a rate closest to the phenylalanine-containing wild-type prepilin. Interestingly, substitution of an asparagine at this position resulted in a turnover rate approximately one-tenth that of wild-type substrate but with a higher affinity for PilD. This finding could form the basis for the design of peptide inhibitors or pseudosubstrates of PilD that may aid in the study of the cleavage reaction.

Prepilin Processing and Biogenesis of N. gonorrhoeae *Pili*

The prepilin of *N. gonorrhoeae* can be efficiently processed by *P. aeruginosa* PilD, and gonococcal membranes contain an enzyme with a prepilin leader-peptidase activity (71). The structural requirements for processing within prepilins of all group A type IV pilins are therefore very likely identical.

Gonococci can produce a truncated form of pilin that is exported from the cell in a soluble form. These S-pilins are processed at a different site within the amino terminus of the mature protein and are smaller than the pilin proteins assembled into pili. Two types of genetic events can lead to truncation of pilin and its export. Mutations that alter the leader-peptide cleavage site, specifically changing the invariant glycine at the −1 position to serine, result in cleavage of ~40 amino acids from the amino terminus and export of pilin from the cell. Alternate cleavage and export of pilin also often accompanies mutations in *pilC*, which encodes a minor protein component of pili (see below). In addition to these mutations, several changes occur in the mature portion of pilin, presumably facilitating cleavage at an alternative site.

Gonococcal outer membranes contain a minor protein of M_r 110,000 that is also present in purified pili (42). The gene for this protein, designated *pilC*, was cloned and sequenced and found to exist as two nonlinked copies, *pilC1* and *pilC2*, in most strains of *N. gonorrhoeae*. However, in the piliated strain MS11 (P+), only the *pilC2* copy is expressed. Insertional inactivation of *pilC2*, but not *pilC1*, prevented the formation of pili but did not abolish expression of the pilin subunits; hence PilC functions in assembly of pili. One class of spontaneous nonpiliated variants is a result of the absence of PilC. PilC is not expressed because of a frameshift mutation in a cluster of G residues located in the region encoding the PilC signal peptide. Reversion back to the piliated phenotype results from the restoration of the reading frame within this G-track. The alteration of piliation by modulating expression of PilC is therefore another mechanism of phase variation. Interestingly, many of the mutants unable to express PilC synthesize a secreted form of pilin (S-pilin) that is generated by alternative processing within the amino-terminal region of the mature polypeptide (43). The reversible PilC-dependent expression also leads to sequence alterations in the expression locus for pilin, and therefore represents another mechanism of antigenic variation. The exact role of PilC in biogenesis of gonococcal pili has not been determined, and a protein that structurally or functionally resembles PilC in other bacteria expressing type IV pili has not been identified as yet.

Biogenesis of Toxin-Coregulated Pili of V. cholerae

The ToxR-coregulated pilin gene (*tcpA*) of *V. cholerae* was originally identified using Tn*phoA* insertions that require ToxR, the transcriptional

regulator of the cholera-toxin operon, for expression (114). The gene encoding TcpA is part of a gene cluster involved in regulation and assembly of the TCP pilus. Other genes in the cluster include *tcpB*, *tcpQ*, *tcpC*, *tcpR*, *tcpD*, *tcpS*, *tcpT*, *tcpE*, *tcpF*, and *tcpJ*, located immediately downstream of *tcpA*, and *tcpH*, *tcpP*, and *tcpI*, located upstream of *tcpA*. These genes are also regulated by the product of the *toxR* gene (Figure 2) (74, 112). Another gene located outside of this cluster, *tcpG*, encodes a product required for efficient biogenesis of TCP pili (81). Although the organization of the *tcp* genes originally suggested some resemblance to the biogenesis functions of the *E. coli* Pap pili operon, the complete sequence of the *tcp* genes revealed little similarity with the Pap pili operon. Therefore, the mechanism of biogenesis of the group B members of the type IV pilin family may be unique.

Two genes required for assembly of TCP encode proteins of known biochemical function. TcpJ is a prepilin peptidase, responsible for the cleavage of the 25–amino acid leader peptide from the precursor of TcpA (44). It is also highly similar to PilD, especially in the cytoplasmic region that contains the active-site cysteines. Because mature TcpA undergoes the same postcleavage *N*-methylation as the other type IV pilins, TcpJ, like PilD, may be a bifunctional enzyme with both protease and methyltransferase activities.

The only gene not part of the *tcp* gene cluster is *tcpG*, which encodes a 24-kDa periplasmic protein. The sequence of TcpG shows striking similarity to several bacterial thioredoxins, *E. coli* DsbA (a periplasmic thiol-disulfide interchange protein), and eukaryotic protein disulfide isomerase (81, 127). All of these proteins have been implicated in mediating thio-disulfide exchange reactions, and the homology of these proteins with TcpG is in the cysteine-containing active centers. Purified TcpG has oxidoreductase activity and probably participates in some chaperone-like function, perhaps controlling folding of TcpA by formation of transient disulfide-linked intermediates or by catalyzing formation of correct disulfide bonds. Interestingly, mutations in TcpG also cause a pleiotropic defect in the extracellular secretion of cholera toxin A subunit and a major protease. These results indicate that the function of TcpG is not restricted to biogenesis of pili, but is also involved in some steps during extracellular protein secretion.

Two additional products of the *tcp* operon share sequence similarity with the biogenesis proteins of *P. aeruginosa*. TcpT is a homologue of PilB (and PilT) as well as other related proteins involved in extracellular protein secretion (see below) and displays a well-conserved nucleotide-binding region. TcpE reportedly has homology to *P. aeruginosa* PilC (45). These similarities with the biogenesis genes of *P. aeruginosa* further reinforce the overall relationship among the type IV pili not only in the primary sequence of the major subunits but in the overall mechanism of organelle biogenesis. Whether

conservation of the export or assembly functions can be extrapolated to the point that they are interchangeable remains to be experimentally determined.

TWITCHING MOTILITY

Most bacteria expressing type IV pili can move on solid surfaces; this is called twitching motility. The ability of type IV pili to undergo reversible depolymerization/assembly of the filament presumably mediates this action. Recently, a gene necessary for twitching motility in *P. aeruginosa* was isolated by restoring twitching motility to a nonretractable, hyperpiliated strain, PAK/2pfs, with a DNA expression library derived from another *P. aeruginosa* prototype wild-type strain, PAO1 (125). This gene (*pilT*) was sequenced, and its 344–amino acid product was shown to share extensive homology with two other *P. aeruginosa* proteins, PilB and XcpR, that are required for biogenesis of pili and extracellular protein secretion, respectively. One of the striking features of these proteins is the presence of two highly conserved domains that contain consensus sequences found in several nucleotide-binding proteins in both prokaryotes and eukaryotes (as discussed above) (115, 121). Further analysis of the PilT amino acid sequence shows that it is largely hydrophilic and lacks both a leader sequence common to exported proteins and stretches of hydrophobic residues that could act as membrane-spanning domains. Thus, PilT is probably a cytoplasmic nucleotide-binding protein, although its role in twitching motility is as yet undefined.

EXPRESSION OF TYPE IV PILIN: CONTROL AT THE TRANSCRIPTIONAL LEVEL

The ability to control expression of the genes encoding structural components as well as proteins involved in assembly of pili is important for several reasons. Formation of functional pili requires interactions between various structural and nonstructural proteins in vastly different ratios, ranging from a few molecules of an enzyme to several thousand copies of the structural subunits. Differential expression of genes assures accumulation of the various proteins in precise relative proportions as the pilus filament is assembled. Expression of pili specifying adhesins and additional bacterial virulence factors results in the evolution of regulatory networks that control gene expression in response to the environmental signals of the host. Finally, because pili are prominent surface structures, they also are occasionally targets of host defenses, and alternating induction and shutoff of pilus expression allows for successful evasion of the immune system. This section discusses selective examples of the mechanism of transcriptional control of expression of type IV pilins.

P. aeruginosa *Pilin*

Johnson et al (40) were first to recognize that the promoter of *pilA*, the pilin structural gene, resembles the promoter for genes transcribed by RNA polymerase containing an alternative sigma factor, σ^{54}, encoded by the enterobacterial *rpoN* gene. The promoters of all members of the type IV group A pilin family contain a conserved motif found in RpoN-dependent promoters, $G_{-24}G...(N_{10})...GC_{-12}$, found upstream of the transcriptional start site (51). The group B pilins lack this sequence and hence are transcribed by the RNA polymerase with the major sigma factor, σ^{70}. The requirement for RpoN in transcription of the pilin gene in *P. aeruginosa* was verified by constructing a strain with an insertionally inactivated RpoN that could not transcribe the pilin gene (38, 38a).

Two additional genes have been identified that encode positive regulators of pilin transcription. A screen of a Tn*5* library of *P. aeruginosa* using a pilin-*lacZ* transcriptional fusion yielded two mutations in linked genes. These genes were cloned by genetic complementation of the transposon mutants. The deduced sequence of one of these regulatory genes, designated *pilR*, belongs to the response regulator class and is homologous in several regions to the prototype of this family, NtrC (38). The most extensive homology between PilR and NtrC occurs at the amino terminus and in a central region. The amino-terminal domain contains conserved aspartic acid residues at positions 11 and 54 and a lysine at position 104 of the 446–amino acid protein (~50 kDa). These conserved residues are found in all members of this class. The central domain of PilR contains two motifs found in a superfamily of ATP-binding proteins (27). In NtrC, this nucleotide-binding domain is required for the endogenous ATPase activity of its phosphorylated form, which is essential for formation of the open complex during initiation of transcription (124). If analogous to other members of the NtrC-family, PilR requires phosphorylation of one of the aspartic acid residues for it to activate transcription of the pilin gene. Mutant forms of PilR, with substitutions at Asp54, cannot function in transcription of the pilin gene (J. Boyd & S. Lory, in preparation).

The second *P. aeruginosa* pilin transcriptional activator gene has been designated *pilS*, and according to sequence homology, it is a sensor or histidine kinase member of the two-component class (J. Boyd & S. Lory, in preparation). The autophosphorylation of a single histidine in the sensor protein, followed by the transfer of the phosphate to the aspartic acid residue of the regulatory protein, relays an environmental signal to the level of gene expression. Mutants of *P. aeruginosa*, in which the histidine at position 115 of PilS was changed to arginine, leucine, or proline, did not synthesize pili. The mechanism of transcriptional regulation mediated by the PilR/PilS pair

seems to resemble the other members of the two-component regulatory family. However, the specific environmental or nutritional conditions that influence the regulation of pilus biosynthesis have yet to be determined for *P. aeruginosa* or any of the other organisms expressing type IV pili.

N. gonorrhoeae *Pilin*

As predicted by the presence of a consensus RpoN promoter sequence upstream of the pilin gene in *N. gonorrhoeae* (*pilE*), the expression of this type IV pilin gene very likely involves RpoN and the cognate regulatory factors. Additional regulatory proteins, which do not resemble the *P. aeruginosa* transcriptional factors, have been described. Two closely linked genes, designated *pilA* and *pilB*, were isolated and shown to act *in trans* to regulate pilin gene expression (111). Initial experiments showed that the *pilB* gene product, in conjunction with the product of *pilA*, decreases pilin expression in *N. gonorrhoeae*, and hence, gonococcal mutants in PilB are hyperpiliated. In *E. coli*, the *pilA* gene product stimulated pilin-promoter activity. However, a *N. gonorrhoeae pilA* mutant could not be obtained, suggesting that this gene has an essential regulatory function in gonococci. Attempts to construct null mutants in both *pilA* or *pilB* by transposon mutagenesis were also unsuccessful. Construction of $pilA^{-}/pilA^{+}$ merodiploid gonococci led to a drastic reduction in the amount of pilin production; taken with the stimulatory effect of PilA on the pilin promoter in *E. coli*, these results suggested that this gene product is also a regulatory protein.

Analysis of the amino acid sequences of PilA and PilB showed similarities to members of the two-component regulatory-system family (109). PilB is homologous to the histidine kinase sensor component, and work with gene fusions to alkaline phosphatase and cellular localization studies show it to be a cytoplasmic membrane protein with both periplasmic and cytoplasmic domains. As with several members of this class of regulatory proteins, the periplasmic domain is very likely to be the sensor, whereas the cytoplasmic domain is the transmitter in an as-yet uncharacterized signaling pathway. The cellular localization of PilB in gonococci is therefore different than that of PilS, its counterpart in *P. aeruginosa*. However, as with PilS, the environmental signals that induce PilB to transmit an activation signal to initiate pilin transcription are unknown.

PilA is homologous to the response-regulator proteins of the two-component family (109). As with PilR of *P. aeruginosa*, gonococcal PilA appears to have DNA-binding capability and also has a carboxy-terminal ATP-binding consensus sequence. However, two characteristics of PilA distinguish it from PilR as well as from most other response regulators. First, the phospho-acceptor residue in all regulatory elements of the two-component family is an

aspartic acid, while PilA contains a glutamic acid at the corresponding position. Second, PilA appears to be an essential gene for *N. gonorrhoeae,* whereas PilR is not necessary in *P. aeruginosa*. PilA therefore most likely controls expression of an as-yet unidentified gene that is essential for gonococcal growth under most laboratory conditions.

V. cholerae *TCP*

The molecular mechanism controlling expression of toxin-coregulated pili of *V. cholerae* involves activities of regulatory elements that coordinately control expression of several other virulence factors. *V. cholerae* mutants in *toxR,* the gene encoding the regulator of the cholera toxin operon, are unable to express pili. Expression of a fusion between *tcpA,* which is the gene for the structural subunit of TCP, and alkaline phosphatase also depends on functional ToxR (114). Genes under *toxR* control include not only the structural subunit gene, but also the remaining linked genes of the TCP operon, as well as the unlinked gene *tcpG*. The genetic organization of the *tcp* accessory genes suggests that a single promoter upstream from *tcpT* directs transcription of all of the remaining *tcp* genes, whereas the gene for *tcpI* is transcribed in the opposite direction. Interestingly, a site of transcriptional initiation, or mRNA processing, has been also identified upstream of *tcpA* mRNA (R. Taylor, personal communication).

Among all of the ToxR regulated genes, only transcription of the *ctx* operon is activated in *E. coli* in the presence of ToxR. This observation led to a search for other transcriptional elements that may act together with ToxR in transcriptional initiation. ToxT was identified as a regulatory factor required for expression of many toxin-coregulated genes, including the genes of the *tcp* operon. ToxT is a 32-kDa transmembrane protein that shares similarity with the members of the AraC family of transcriptional activators (15, 73). It may act as a site-specific DNA-binding protein with several unusual features. Transcription of *toxT* depends on ToxR, and hence ToxR exerts its activity on many toxin-coregulated genes, including the *tcp* locus indirectly, by regulating *toxT*. Interestingly, the gene for ToxT is within the *tcp* operon, and it may therefore be under autoregulatory control from regulatory sites that precede this operon. The products of *tcpH* and *tcpI* apparently encode additional regulators of the *tcp* operon, although their mechanism of action is not known (113).

The *tcp* genes respond to environmental signals transmitted to the various structural and biogenesis genes of *V. cholerae* pili via the ToxT-ToxR cascade. The intramembrane disposition of ToxR and its ability to recognize promoters of regulated genes suggests that this protein transmits signals from the periplasmic side of the inner membrane and effects transcription via its cytoplasmic domain by binding regulatory sites near promoters, including the

one near *toxT*. The environmental conditions that control expression of *tcp* genes include osmolarity, temperature, and pH. That these signals control coordinate expression of various cell-associated and extracellular virulence factors may reflect the different environments that *V. cholerae* experiences prior to and during colonization of the lower intestinal tract (64).

RELATIONSHIP OF TYPE IV PILI AND PROTEIN EXPORT

Phenotypic characterization of *P. aeruginosa* mutants in the pilus biogenesis determinants *pilB, pilC,* and *pilD* revealed that PilD functions not only in processing of prepilin, but that it has a second role in extracellular protein secretion. Mutations in *pilD* lead to periplasmic accumulation of exotoxin A, elastase, alkaline phosphatase, and phospholipase C—enzymes that are actively exported from wild-type *P. aeruginosa* (105). The secretion signal of exotoxin A, found in the periplasm of *pilD* mutants, is cleaved. These findings suggested that PilD has additional substrates in the bacterial cell, and these substrates are essential components of the extracellular-secretion apparatus. Independently, Bally et al (3) isolated a gene essential for extracellular protein export from *P. aeruginosa*. The product of this gene, XcpA, is identical to PilD, hence confirming the central role of PilD in protein export as well as biogenesis of pili.

In order to identify substrates of PilD, a series of degenerate oligonucleotides corresponding to possible codons spanning the prepilin cleavage site were designed and used as probes to screen cloned DNA from sites of transposon insertions in pleiotropic export-deficient *P. aeruginosa* mutants. The probes identified a region of DNA containing four genes that encode proteins with a high degree of similarity to prepilin (72) (Figure 3). These proteins, termed PddABCD (for PilD-dependent proteins), are required for extracellular secretion and are substrates of PilD (106; D. N. Nunn & S. Lory, submitted). Analysis of a cluster of genes originally isolated in export-deficient *P. aeruginosa* mutants and designated *xcp* led to identification of four genes (*xcpTUVW*) (4) that are identical to *pddABCD*.

The similarity between type IV pilin and proteins of the extracellular secretion apparatus is not restricted to *P. aeruginosa*. Several genes have been identified during the past two years that are homologues of type IV prepilins, and with a single exception, they are part of a protein-export machinery. All of these bacteria also express a protein similar to PilD. These include the pilin homologues PulGHIJ and the leader peptidase/methylase PulO in *K. oxytoca* (85). Extracellular secretion of degradative enzymes in the plant pathogens *Erwinia chrysanthemi, Erwinia carotovora,* and *Xanthomonas campestris,* and in *Aeromonas hydrophila,* involve similar genes, organized in clusters,

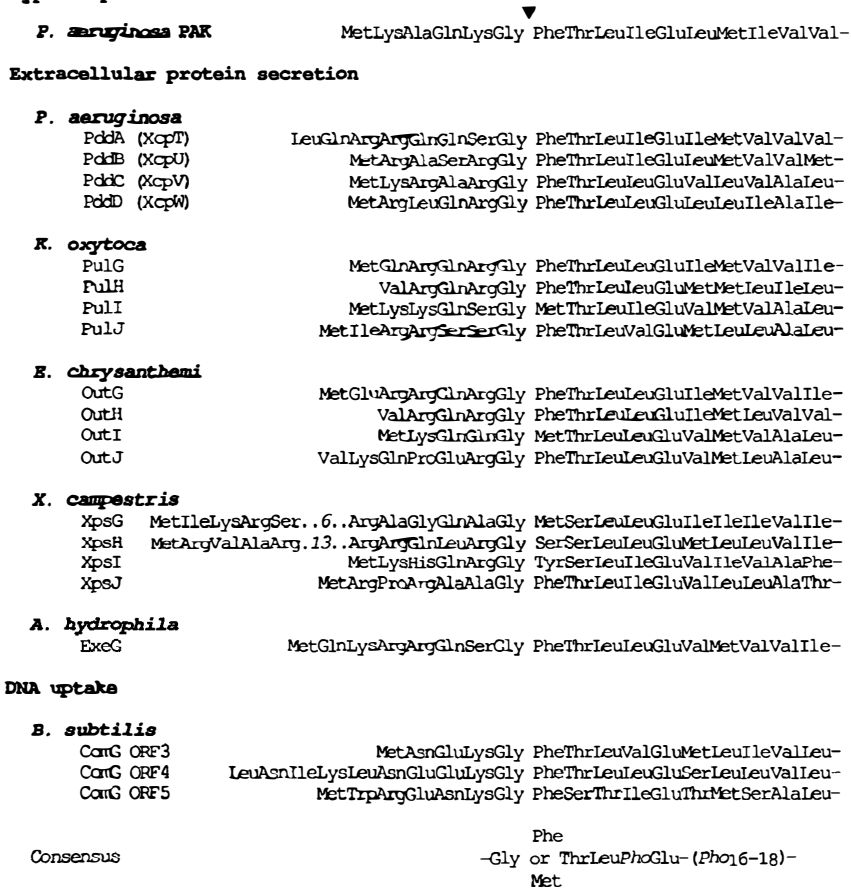

Figure 3 Comparison of the amino termini of the precursors of type IV pilin–like proteins involved in extracellular protein secretion or DNA uptake. These are compared with the type IV pilin from *P. aeruginosa* PAK (41). The upside-down triangle denotes the putative leader peptide-cleavage site. The sequences for these proteins were first described in references: *P. aeruginosa* PddABCD/XcpTUVW (4, 72); *K. oxytoca* PulGHIJ (85); *Erwinia chrysanthemi* OutGHIJ (31, 56); *Xanthomonas campestris* XpsGHIJ (21, 35); *Aeromonas hydrophila* ExeG (8); *B. subtilis* ComG (ORF3,4,5) (1, 9).

several of which encode type IV pilin–like proteins (Figure 3) (8, 20, 21, 31, 35; G. P. Salmond, personal communication).

The genes responsible for extracellular protein export are usually clustered and in some instances organized into a single operon (84). This region contains not only genes homologous to type IV pilin, but other functions related to

biogenesis of type IV pilins. For example, in *P. aeruginosa,* significant homologies can be found among products of *xcpR* and *pilB* and *pilT* (4, 125). Also, PilC of *P. aeruginosa* is similar to XcpS (4). Corresponding genes can be found in *K. oxytoca, Erwinia* sp., and *X. campestris.* These findings suggest that the genes found in the export cluster specify determinants that facilitate assembly of organelles resembling pili by ordered assembly of the four heterologous subunits encoded by adjacent genes. The existence of a complex composed of the four pilin homologues has not been identified in the bacterial envelope.

The only homologues of type IV pilins not associated with extracellular protein secretion are encoded by genes in *B. subtilis* required for the competence state and DNA uptake. Clearly, the bifunctional enzyme PilD and its homologues have a central role not only in type IV pilus biogenesis, but also in macromolecular transport across cell membranes.

CONCLUDING REMARKS

The discovery during the past decade that a diverse group of pathogenic bacteria express pili related by their structure and their mechanisms of regulation and assembly has provided a very useful model to study expression of virulence factors. The role of type IV pili in virulence is now firmly established, and studies of pilus biogenesis and function are aimed not only at understanding the mechanism of bacterial pathogenesis better but also at exploring the possibility of novel approaches to prevention and therapy of infectious disease.

Numerous questions remain to be answered regarding the role of pili in pathogenesis. Specifically, the precise molecular nature of the interaction of an adhesive component of pili with host receptors is not completely clear. Complicating these studies is the observation that the surfaces of virtually all bacteria that express type IV pili also possess other adhesins. The receptor-binding contribution of the pilin subunits and the range of receptors recognized by the different adhesins is therefore difficult to measure. Promising developments in bacterial genetics of many bacteria with type IV pili should allow construction of isogenic mutants that cannot express individual adhesins. Such strains will be extremely useful in dissecting the complex interactions of bacteria with their hosts.

The processes of secretion and assembly of pili have been studied primarily using genetic methods. In the future, efforts must be directed towards understanding the mechanisms of biogenesis and identifying the precise function of products of individual biogenesis genes in the formation of pili. Although some of the proteins have known biological functions, such as PilD of *P. aeruginosa* and TcpJ and TcpG of *V. cholerae,* the products of the

majority of genes identified by their nonpiliated phenotypes have no known functions. No information is available at this time on the mechanisms that allow subunits of type IV pilin to cross the inner and outer membrane and how the pili are anchored in the bacterial envelope. Based on the unique properties of the type IV pili, answers to these questions will very likely provide insights on previously undescribed basic biological processes of membrane targeting and macromolecular assembly.

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