Keywords
pilus, fimbriae, assembly system, secretin, type IVa, type IVb

Abstract
Type IV pili (T4P) are one of the most common forms of bacterial and archaeal surface structures, involved in adherence, motility, competence for DNA uptake, and pathogenesis. "Pseudomonas aeruginosa" has emerged as one of the key model systems for the investigation of T4P structure and function. Although its reputation as a serious nosocomial and opportunistic pathogen is well deserved, its facile growth requirements and the ready availability of molecular tools have allowed for rapid advances in our understanding of how T4P are assembled, their contributions to motility, biofilm formation, and virulence, and their complex regulation. This review covers recent findings concerning the three different types of T4P found in "P. aeruginosa" (type IVa, type IVb, and Tad) and provides details about the modes of translocation mediated by T4aP, the architecture and function of the T4aP assembly system, and the complex regulation of T4aP biogenesis and function.
INTRODUCTION

Watching *Pseudomonas aeruginosa* spread rapidly across a surface by twitching motility is endlessly fascinating (Supplemental Movie 1; follow the Supplemental Material link from the Annual Reviews home page at http://www.annualreviews.org). This form of bacterial movement was hypothesized by Bradley (29) to result from the repeated extension, tethering, and retraction of long protein fibers called type IV pili (T4P). He showed that the shortening of T4P after attachment of pilus-specific bacteriophages resulted in trafficking of phage particles to the bacterial cell surface (28). A wide variety of bacteria and archaea have since been found to use T4P for functions ranging from DNA uptake to electron transport (11, 141). Because they are genetically amenable human pathogens, *Neisseria* and *Pseudomonas* species—particularly *P. aeruginosa*—have emerged as the favored model organisms for T4P structure and function investigations. Other well-studied but substantially more complex systems (reviewed in Reference 113) include that of the soil bacterium *Myxococcus xanthus*. In this review, the rapid progress that has occurred in the field of twitching motility over the past decade since the last major coverage of this topic (107, 151) is discussed, with a particular focus on *P. aeruginosa*. For additional information, readers are directed to excellent reviews covering various aspects of T4P assembly, structure, and function (3, 11, 12, 30, 41, 42, 67, 69, 79, 120, 135, 137, 138).

T4a, T4b, AND Tad SUBCLASSES OF TYPE IV PILI

Like other kinds of pili and fimbriae, T4P provide the ability to adhere to chemically diverse surfaces—from glass and stainless steel to mammalian cells—and promote bacterial cell aggregation involved in microcolony formation and virulence. However, T4P are unique in mediating flagellum-independent motility (126). There are two major subfamilies of T4P, type IVa and
Type IVb (Figure 1). Type IVa pili (T4aP) are a relatively homogeneous and broadly distributed subtype (135), whereas type IVb pili (T4bP) are a more heterogeneous group. The T4bP subfamily is common in enteric species, such as *Vibrio cholerae* (150) and enteropathogenic *Escherichia coli* (EPEC) (157), and on plasmids and other mobile genetic elements (86).

The T4b subfamily includes a monophyletic class called the tight adherence pili (Tad, or Flp pili), first described in *Aggregatibacter* (previously *Actinobacillus*) *actinomycetemcomitans* (77). Tad pili are found in a wide variety of environmental species such as *Caulobacter crescentus* (156). The Flp pilin subunit is much smaller (7–8 kDa) than the typical T4a or T4b pilin subunit (∼15–20 kDa). Despite the differences in size, all three pilin subtypes have a highly conserved, hydrophobic α-helical N terminus (42) that contains a characteristic type III signal sequence (182). Unlike signal sequences recognized by signal peptidases I or II, both of which act at the exterior of the cytoplasmic membrane, the type III motif is cleaved by a dedicated prepilin peptidase (121) at the cytoplasmic face of the membrane. Although T4a and T4b pili were traditionally classified by subtle differences in their major pilins (41), it is now clear that each subclass has a distinct assembly system whose components are diagnostic (Figure 1) and see below).

To date, *P. aeruginosa* is the only species in which T4aP, T4bP, and Tad systems in a single strain have been reported (21, 31, 49). The T4aP- and Tad-encoding genes are common to all *P. aeruginosa* genomes sequenced thus far (173), whereas T4bP are found in strains that have *P. aeruginosa* pathogenicity island 1 (PAPI-1) or related elements such as pKLC102 (31, 178). Although each of the three T4P subtypes has a separate assembly system (Figure 1), the *P. aeruginosa* T4bP system is dependent on the T4aP prepilin peptidase PilD for processing subunits prior to their assembly (31). The Tad system encodes its own prepilin peptidase, FppA, specific to the Flp subunit (49).
T4b and Tad pili are not typically associated with motility, and many systems lack an ortholog of the PilT ATPase involved in pilus retraction. However, at least some T4bP systems may have retraction capabilities associated with other phenotypes. In the case of C. crescentus, the disappearance of Tad pili that occurs with stalk biogenesis was suggested to occur via retraction, since neither pili nor their subunits were detected in the medium (96). EPEC uses T4bP to form bacterial aggregates on intestinal epithelia (the localized adherence, or LA, phenotype). Disaggregation of the microcolonies—presumably by pilus retraction—is important for virulence, as inactivation of the PilT ortholog BfpF reduced pathogenicity in human volunteers (24). A recent study also showed that BfpF function is important for disruption of host cell tight junctions and for promoting the close contact between bacterial and host cells needed for engagement of the type III secretion system. The T4bP system in P. aeruginosa has a PilT homolog (PilT2) (Figure 1) and is self-transmissible via conjugation (31); retraction of the T4b pilus may be required for productive mating pair formation.

CRAWLING, WALKING, SWARMING, AND SLINGSHOT MOTILITIES

Many (but not all) T4aP-expressing species, including Neisseria spp., Dichelobacter nodosus, and P. aeruginosa, exhibit pilus-mediated twitching motility (referred to as social motility in M. xanthus). Some T4aP-expressing species that lack macroscopic twitching motility when examined under the in vitro assay conditions used for P. aeruginosa may still have limited motility visible by light microscopy. Twitching occurs on moist surfaces of moderate viscosity, equivalent to that of 1% agar. Although individual cells are capable of movement, it is common to see them moving in rafts, groups of cells preferentially aligned along their long axes (149). Individual cells often snap into alignment with the group when they encounter a raft, contributing to the jerky appearance of twitching motility. It is surprising that even though cells typically move as a group, the exact requirements for an individual cell to participate have not been established. Does each cell need to have pili, or only a subset? Do all the cells need to be motile, or can some be nonmotile? These are simple questions, but important in understanding how twitching cells are coordinated.

The exact distance traveled by twitching cells depends on both extrinsic and intrinsic factors. Extrinsic factors include medium (nutrient) composition (75), viscosity, and hydrophobicity of the surface (149), and intrinsic factors include the amount of pili produced and their retraction rates, and the production of surface-tension-reducing surfactants (129). When a common 1% Luria-Bertani agar stab assay is used, P. aeruginosa strains can form a thin, radial twitching zone ∼2 cm in diameter around the point of inoculation after 20 h of incubation, a velocity of ∼1 mm h⁻¹. If the average P. aeruginosa cell is ∼2 μM in length, this translates to around 500 cell lengths per hour, although the actual distance traveled is likely to be considerably farther, as the bacteria do not travel in a straight line.

T4P are so thin (<8 nm in diameter), they cannot be visualized readily by light microscopy, and thus it was initially difficult to conclusively link pilus retraction to motility. Evidence of pilus retraction by live cells was first provided by optical tweezers studies showing that Neisseria spp. connected by their pili to beads could reduce the distance between cells and beads while generating substantial forces in the ∼100 pN range (106, 111). Even greater forces (∼150 pN) were found when similar studies were performed with M. xanthus, potentially because it has multiple retraction motors (37).

In 2001, Skerker & Berg (155) published a seminal study in which they used total internal reflectance microscopy to image live, nonflagellated P. aeruginosa labeled with a fluorescent, aminospecific dye. By using this method, it was possible to directly observe single, highly flexible pilus fibers extending from individual cells and exploring the adjacent surface before adhering to it via the pilus tip. Attached pili became taut and shortened, resulting in translocation of the cell body.
In addition to supporting the hypothesis that twitching motility results from pilus retraction, the authors showed that retraction of single pilus fibers did not occur in a coordinated fashion; each fiber appeared to retract independently of the others. Also, newly extended pili appeared to be as brightly fluorescent as those already on the surface, implying that they were assembled—at least to some extent—from fluorescent subunits recycled from pili that were disassembled upon retraction after the initial labeling step.

Subsequent studies demonstrated that even though pili can be retracted individually, the cooperative retraction of several bundled fibers generates more substantial forces (in the nanonewton range, versus piconewton range, for a single pilus) (23) and allows cells to travel distances that exceed the length of individual pili. To correlate the number of pilus fibers per cell with the distance traveled by an individual cell, Holz et al. (73) used light microscopy to monitor the motility of individual live cells on electron microscopy grids, followed by rapid fixation, negative staining, and enumeration of the average number of pili per cell. By genetically manipulating the number of pili produced per cell, they showed that bacteria with the most pili traveled farther than bacteria with fewer pili, because of the sharing of forces among multiple fibers. The angles at which individual fibers bind—relative to the cell axis—and the comparative retraction forces on each ultimately affect the direction in which the cell body moves.

Other Forms of T4aP-Mediated Motility

Although twitching motility is the best-characterized type of movement associated with T4aP, a number of other T4aP-dependent modalities have been described for P. aeruginosa, including swarming, walking, and slingshot motilities. Swarming motility—a complex phenotypic adaptation (127, 180) that affects a number of traits—occurs on medium that is less viscous than standard twitching motility medium (0.4–0.7% agar versus 1% for twitching). Swarming motility is characterized by the formation of elaborate dendritic patterns by the swarming colony (117) (Figure 2). Under specific nutrient conditions, swarming motility requires T4aP (89, 128), but provision of specific carbon sources including glutamate, glucose, or succinate restores swarming—in some cases, hyperswarming—of pilin mutants (153). Swarming is controlled in part by the pilus-related chemotaxis system (below), as point mutations in the chemosensory protein ChpA decrease or modulate swarming (98).

A novel type of T4aP-mediated walking motility was described recently (39, 57). The investigators developed customized particle-tracking algorithms to quantify the movement of large numbers of individual cells during videomicroscopy of P. aeruginosa in flow cells. Two distinct modes of T4aP-related motility were observed: crawling motility, the archetypal twitching mode in which cell bodies are oriented parallel to the surface, and walking motility, in which cell bodies are oriented at right angles to the surface plane and attached by pili extending from the surface-proximal pole. In crawling mode, the cells moved more slowly and were less likely to change direction (high directional persistence), whereas cells in walking mode moved more quickly and were more likely to ramble on the surface (low directional persistence) (Figure 3). The horizontal and vertical states were strongly favored over intermediate states, although cells frequently switched between the two orientations in a flow-independent manner. Use of a flow cell was key to observing walking motility, affording cells the opportunity to orient vertically.

The most recently identified mode of T4aP-mediated motility, slingshot motility, contributes significantly to the overall distance traveled by a cell and to its ability to change direction on a surface (80). By separately tracking the leading and trailing poles of cells crawling along a surface, Jin et al. (80) saw that most of the time during twitching (crawling) motility, the leading and trailing poles of a cell moved in the same direction, parallel to the long axis of the cell, at roughly
Wild type

Figure 2
Role of Type IVa pili (T4aP) in swarming and sliding motility. On medium of intermediate viscosity, wild-type *Pseudomonas aeruginosa* is capable of swarming motility, forming elaborate dendritic colonies (top left). Pilated mutants lacking flagella (*fliC*) are nonmotile under these conditions (bottom left), whereas flagellated mutants lacking type IV pili (T4P) (*pilA*) show aberrant swarming motility (top right). Mutants lacking both T4P and flagella (*pilA, fliC*) can move by sliding motility (bottom right), suggesting that expression of T4P hampers sliding in the *fliC* background. Figure reproduced from Reference 117 with permission.

Figure 3
Type IV a pili (T4aP)-mediated crawling versus walking motility. Cells exhibiting crawling motility (blue trace), the mode traditionally associated with twitching, are oriented parallel to the surface and change direction infrequently. Cells exhibiting walking motility (red trace) are oriented perpendicular to the surface and change direction frequently. Figure reproduced from Reference 39 with permission.

Changes may still occur before final publication online and in print.
surfactant production but was inhibited by T4aP expression. The authors suggested that flagella promote swarming by overcoming the adhesive interactions generated by T4aP. Similarly, Taylor & Buckling (162) showed that P. aeruginosa lacking T4aP traveled farther by swimming motility than isogenic mutants lacking the PilU retraction ATPase, presumably because the coexpression of nonretractile pili with flagella reduces the efficiency of flagellum function. Li et al. (100) showed recently that attachment of the polar Tad pili of swimmer cells of C. crescentus to surfaces resulted in the mechanical jamming of the flagellum at the same pole, a signaling event triggering the release of exopolysaccharide adhesins that subsequently promote irreversible surface interactions.

**TWITCHING MOTILITY: WHAT’S IT FOR?**

Although most bacterial pili and fimbriae are used for adherence, the ability to retract provides bonus functions: bringing the cell body into intimate contact with a surface, allowing migration along a surface away from the initial point of contact or toward an attractant, repositioning cells with respect to one another (differentiation within a biofilm), and helping cells efficiently escape from surfaces when necessary.

**Arrivals and Departures**

The primary function of twitching motility is likely exploration of surfaces, using the crawling, walking, swarming, and skidding modes described above. If the environment is unsuitable, however, a means of escape is required. In collaboration with flagella, T4aP are crucial to the dispersal of bacteria from surfaces in a coordinate series of events called a launch sequence by Conrad et al. (39). Cells tethered to the surface by their flagella and rotating rapidly moved from shallow (∼30°) to steeper (∼70°) angles in a T4aP-dependent manner, eventually breaking loose and swimming away. In contrast, flagellated cells lacking pili were unable to reorient themselves and detach. The inability of nonpiliated cells to detach efficiently from the surface following initial attachment resulted in profound differences in the architecture of biofilms arising from the colonizers (Figure 4).

**Biofilm Development**

T4P are important adhesins, promoting initial attachment to a variety of chemically diverse surfaces. They were one of the first factors identified in genetic screens looking for traits important for P. aeruginosa biofilm formation (124), participating in both surface attachment and microcolony formation. Chiang & Burrows (33) showed that although the adhesive capacity of T4aP was important in establishment of biofilms in flow cells, twitching motility played a role in development of biofilm architecture. Mutants lacking the retraction ATPase PilT formed thick, undifferentiated biofilms compared with those that were able to twitch. Those data are consistent with the study by Conrad et al. (39) in which nonpiliated cells formed aberrant biofilms, showing that fully functional T4aP are important for structuring a biofilm. It would be interesting to further study the contribution of twitching to biofilm architecture using strains in which PilT expression is inducible to determine whether its latent expression in preformed biofilms of abnormal architecture would lead to their remodeling.

Elegant studies by the Tolker-Nielsen group (15, 87, 88) shed further light on the specific role that T4aP play in biofilm differentiation. They grew biofilms of yellow fluorescent protein–labeled wild-type P. aeruginosa mixed with cyan-labeled nonpiliated cells. The resulting biofilms had the typical mushroom-like architecture of mature microcolonies separated by water channels,
Figure 4

Effect of type IVa pili (T4aP)-mediated detachment on biofilm architecture. *Pseudomonas aeruginosa* uses its T4aP to orient the cells in such a way that they can detach effectively from surfaces using their flagella. Mutants lacking T4aP (pilA mutant, left) are proficient surface colonizers but deficient in detachment, forming large clumps of cells. In contrast, the detachment-proficient wild type (right) is more uniformly distributed on the surface. Bacteria lacking flagella (fliM mutant, inset) are poor surface colonizers. Figure reproduced from Reference 39 with permission.

but there was a startling spatial separation of the two cell types. The nonpiliated mutants were concentrated in the stalk portion of the microcolonies, and the wild-type parent cells formed the majority of the cap portion (88), suggesting that the wild-type cells used twitching to climb a mound of their nonmotile brethren. Further studies using mutants in the Pil-Chp T4aP regulatory system (below), as well as mutants lacking flagella or impaired in flagellar chemotaxis, showed that altering pilus function caused formation of atypical caps (in the case of pilH and chpA mutants) and that flagella are also necessary for formation of normal microcolony architecture (15). Although they concluded that twitching had only a minor role in cap development, they did not formally test its contribution using a pilT mutant.

A clear picture of twitching motility’s participation in biofilm development is complicated by the fact that excess twitching can impair biofilm formation in the first place. This delicate balance is best illustrated by studies in which the nutrient content of the medium has been altered, affecting twitching motility. For example, Singh et al. (154) showed that chelation of iron by lactoferrin increased twitching motility, causing a commensurate decrease in biofilm formation. This phenotype is due partly to upregulation of rhamnolipid biosynthesis, a biosurfactant that increases twitching and alters biofilm architecture (61).

**Twitching Motility and Pathogenicity**

Because T4P help establish initial contact with a host, they are important virulence factors for many species including *P. aeruginosa* (65), an opportunistic pathogen of fungi, worms, plants, animals, and humans. T4P are deployed in the early or acute phase of infection but are frequently lost owing to downregulation or mutation in chronic infections such as cystic fibrosis (105). In the majority of reports, the specific contributions of T4P-mediated adhesion versus twitching motility have not been clarified, because it is common practice to compare the pathogenicity of...
wild-type strains to mutants lacking pili. Below, I focus on the particular role of twitching motility in virulence as demonstrated in studies using retraction-deficient mutants.

Comolli et al. (38) showed that the PilT and PilU retraction ATPases were important for \textit{P. aeruginosa} cytotoxicity against epithelial cells and for virulence in vivo. Surprisingly, although such mutants are described as hyperpiliated, they found fewer bacteria associated with the epithelial cells compared with mutants lacking pili. This finding implies that additional adhesins on the cell surface need to be engaged to stabilize host-pathogen interactions, and that lack of pilus retraction may prevent those necessary contacts. The dependence of cytotoxicity on pilus retraction was based on the requirement for close apposition of the contact-dependent type III toxin secretion system with the host cell membrane, to allow efficient delivery of effectors (38). Strong evidence for the involvement of twitching motility in \textit{P. aeruginosa} pathogenesis came from the Fleiszig group (181), who showed that mutants lacking PilT or PilU were impaired in corneal infection models. They subsequently demonstrated in vitro using multilayered corneal epithelium that twitching motility mutants were impaired in translocation across the epithelium and in escape from infected cells, phenotypes similar to mutants lacking pili altogether (2).

A recent in vitro evolution study looked at the effects of selecting \textit{P. aeruginosa} strains for the traits of increased twitching or increased swimming motility (163). The two modes of motility were antagonistic, as strains with increased twitching had reduced swimming compared with the parent and vice versa. Strains selected for increased twitching motility (via undefined mechanisms) grew to higher densities in an acute waxworm infection model compared with strains selected for increased swimming. Because the mode of inoculation— injection—bypassed the initial steps of colonization in which T4P normally function, it is unclear whether increased twitching or decreased swimming (a metabolically expensive trait) provided a growth advantage to the host.

Beyond \textit{P. aeruginosa}, a role for twitching motility, or at least for retractile T4P, in virulence is best supported by numerous studies using \textit{Neisseria} retraction-deficient mutants. \textit{N. gonorrhoeae} is an obligate human pathogen and a master manipulator of host signaling cascades. Formation of adherent microcolonies on human epithelia and subsequent T4P retraction, which generates forces in the 70 pN range (122), cause a number of changes in host cells, including recruitment of cytoskeletal and signaling proteins to a cortical plaque beneath the bacteria (54, 110, 168). \textit{N. gonorrhoeae} downregulates proapoptotic and upregulates cytoprotective pathways in epithelial cells in a PilT-dependent manner (72, 74), and a recent study by Dietrich et al. (51) confirmed that the previously reported activation of NFκB by the pathogen was enhanced by T4P retraction. The phenotype was recapitulated in the presence of \textit{pilT} mutants by imposing a shear force on the human cells, confirming early reports (110) that mechanical forces generated by pilus retraction modulate host signaling.

For the T4aP-expressing sheep pathogen, \textit{Dichelobacter nodosus}, Han et al. (66) showed that mutants lacking either PilT or PilU were avirulent and that this deficiency was related specifically to loss of twitching motility. They noted an interesting dichotomy in the two mutants; although both were nonmotile and nonpathogenic, the \textit{pilU} mutant continued to secrete proteases, a T4P-dependent trait in this species. Functional differences between the PilT and PilU ATPases have been noted in a number of species (22, 33–35, 132, 152, 172), suggesting that these proteins have discrete roles despite the apparent similarities between the proteins (below) and some of their mutant phenotypes.

**T4P as Antennae?**

One intriguing but underexplored possibility is that in addition to providing motility, T4P can act as antennae to sense and respond to the bacterial environment. Because of their length (up to...
several microns) they can act at a distance from the cell body but are intimately connected to it via their assembly systems (below). It is not hard to imagine that T4P could transduce to the bacterium a wealth of information about the physical and even chemical nature of the surfaces to which they become attached. The tactile response of a softer versus harder surface when pilus retraction occurs could provide clues that could inform bacterial behavior. In addition to the retraction-related Neisseria host-signaling manipulation studies described above, there is evidence that T4P of M. xanthus can detect bacterial molecules such as exopolysaccharides and respond by retracting (25). The T4P of Geobacter sulfurreducens can conduct electrons released from the respiratory chain to extracellular acceptors (104, 141), making it possible to envision the reverse scenario, in which bacteria use their T4P to receive information about the electrochemical properties of nearby surfaces.

THE TYPE IV PILI MACHINE AND ITS ENGINE

Overview of the T4P Assembly Machinery

From an evolutionary standpoint, the T4P machinery is ancient, present in gram-positive (including the oldest eubacterial class, Clostridia), gram-negative, and archaeal species (135, 164). It has been appreciated for some time that specific T4P system components share sequence similarity with those of the type II secretion system (T2SS) (134). However, recent structural studies of key components have revealed further unanticipated similarities, even between proteins with little to no sequence identity. Because of space limitations, a detailed discussion of structural findings is not possible here. For further information on the structural and functional similarities between T4P and T2SS assembly systems, see References 12, 67, 69, 90, 134, and references therein.

In P. aeruginosa, most of the genes involved in pilus biogenesis were originally identified via mutant screens (48, 107, 108). Strains that exhibited aberrant motility were further examined for susceptibility to pilus-specific bacteriophages. Mutants deficient in pilus biogenesis or retraction typically exhibit resistance to phage killing. However, paradoxical phenotypes were occasionally observed, in which piloted but nonmotile mutants (e.g., pilU) (172) or even apparently nonpiliated mutants (e.g., fimV) (148) remained susceptible to pilus-specific phages, suggesting that there were intermediate phenotypes between the completely nonpiliated and the retraction-deficient hyperpiliated forms. These comprehensive surveys laid the foundation for detailed molecular, biochemical, and structural investigations of components involved in twitching motility in P. aeruginosa. The organization of the genes encoding the T4a assembly system and regulatory proteins is shown in Figure 5, and a list of proteins involved in biogenesis, twitching motility, and regulation of T4P function is provided in Supplemental Table 1. Although many of these components are conserved in other T4P-producing species (reviewed in Reference 135), others are unique to P. aeruginosa.

The T4a pilus and its assembly apparatus can be envisioned as four interdependent subcomplexes that together span the entire cell envelope, with most components located in the inner membrane (Figure 6). The outer membrane subcomplex, which is absent in gram-positive systems, consists of the secretin, a massive (>1 MDa), highly stable dodecamer of PilQ subunits, and its lipoprotein pilotin, PilF, composed entirely of protein-protein interaction motifs called tetratricopeptide repeats (91). PilF is responsible for correct localization and oligomerization of PilQ in the outer membrane, as removal of its lipidation site causes PilQ to multimerize aberrantly in the inner membrane (91). The secretin provides an outer membrane channel for the pilus fiber to exit the cell (175).
Figure 5
Genomic context of type IVa pili (T4aP) genes in reference strain PAO1. Gene names are listed on the arrows, and the corresponding four-digit PA number (PAxxxx) is shown above each gene (173). Genes encoding structural components are colored yellow, and genes encoding regulatory components are colored purple. Genes that are associated with known T4aP genes but lack a pilus-related phenotype are colored gray. PAO1 encodes a group II pilin; the four alternate pilins and their corresponding accessory genes (95) are boxed at the left.
Figure 6
Structures of type IVa pili (T4aP) assembly subcomplex components. The T4aP assembly system comprises four interacting subcomplexes. The outer membrane subcomplex (PilF and PilQ) is shown in dark gray; the inner membrane motor subcomplex (PilB, PilC, PilD, PilT, and PilU) in light gray; the alignment subcomplex (PilM, PilN, PilO, PilP, and FimV) in medium gray; and the pilus subcomplex [the minor pilins (mp) FimU, PilV, PilW, PilX, and PilE and the major pilin, PilA] in white. Structural advances in the T4P and type II secretion systems reveal the architecture of specific components, shown in the surrounding panels. (a) The N-terminally truncated group V pilin from strain Pa80110594, PDB code 3JYZ (119). (b) Full-length PilF from strain PAO1, PDB code 2HO1 (91). (c) C-terminal fragment of PilP from strain PAO1, PDB code 2LC4 (161). (d) The periplasmic fragment of PilO (red, PDB code 2RJZ) modeled as a heterodimer with the predicted periplasmic fragment of PilN (green) (144). (e) The N-terminal cytoplasmic domain of PilC from Thermus thermophilus, PDB code 2WHN (83). (f) A cytoplasmic fragment of EspL (equivalent to PilM, green) and its interaction partner EspE (equivalent to PilB, red), PDB code 2BH1 (1). (g) Two monomers of PilT from strain HA101 (yellow and blue) with bound 5'-adenyl (β,γ-methylene)diphosphonate (AMP-PCP) (green), PDB code 3JVV (116). (h) PilM (red) from T. thermophilus bound to an N-terminal peptide from PilN (green), PDB code 2YCH (82).
Both the motor and alignment subcomplexes are located in the inner membrane. In *P. aeruginosa*, the exact composition of the T4aP motor subcomplex has not been defined but, on the basis of available studies and evidence from related systems, potentially comprises PilB, PilC, PilT, and PilU (34, 170, 172) (Figure 6), as well as regulatory proteins (below). PilB is an hexameric ATPase of the large VirB11 family (35) and is predicted to convert chemical energy from ATP hydrolysis to mechanical energy required for pilus assembly. PilT and PilU are PilB-like ATPases required for pilin depolymerization. PilC is a three-pass membrane protein with two large cytoplasmic domains, each predicted to contain a bundle of six α-helices (83). Aside from the pilins, PilB and PilC are arguably the most readily identifiable and highly conserved components in T4P, T2S, DNA-uptake, and archaeal motility systems, presumably composing the minimal functional unit of the motor (11, 134, 137). PilD, a membrane-bound aspartyl protease that processes the type III signal sequence at the pilins’ N termini, and N-methylates the new terminus (160), might be transiently or permanently associated with the motor subcomplex on the basis of its genetic linkage to PilC in a number of systems and its essentiality for pilus biogenesis. Although the Tad pilus system lacks a retraction ATPase, it has two PilC homologs (21) (TadB and TadC) (Figure 1). It is possible that interaction of the sole TadA ATPase with one platform protein could promote pilin polymerization (below), and interaction with the other could induce depolymerization.

The alignment subcomplex is, as the name implies, proposed to physically connect the inner membrane motor and outer membrane subcomplexes to ensure that the growing, highly flexible pilus is positioned correctly for egress as it begins to extend through the periplasm from its assembly site. In addition, evidence from the related T2SS suggests that components of the alignment subcomplex interact directly with subunits (63), which may increase their local concentration around the assembly site, and participate in gating of the secretin (12). The components of the *P. aeruginosa* T4aP alignment subcomplex include the broadly conserved PilMNOP proteins that are encoded with the PilQ secretin monomer (135), and potentially the peptidoglycan-binding protein FimV (166) (Figure 6). Interestingly, components of the alignment subcomplexes from the T4a, T4b, and Tad pilus systems (Figure 1) and the T2SS are the least conserved in terms of sequence and number, but emerging structural data have revealed some conservation in their architectures (12).

PilM is a cytoplasmic, actin-like protein bound to PilN via the latter’s cytoplasmic N terminus (82). Together, PilM and PilN form the structural equivalent of GspL, an alignment complex protein in the T2SS (144). The PilN and PilO proteins have a similar domain organization, in that both are predicted to have short cytoplasmic N termini, a single transmembrane domain, and a periplasmic C terminus with a long coiled-coil region followed by a ferredoxin-like fold (144). The periplasmic domains of PilN and PilO form highly stable heterodimers in vitro and are dependent on one another for stability in vivo unless PilO is overexpressed, in which case it forms stable but nonfunctional homodimers (13). Although interactions between PilMNO and PilC that would support the proposed alignment function have not been described, GspL and the T2S platform protein GspF interact with one another, as well as with GspM (equivalent to PilO) (144) and GspE (equivalent to PilB) (8, 139). PilP is an inner membrane lipoprotein with a long unstructured N terminus followed by a β-sandwich domain (62, 161). Recent biochemical studies showed that PilP forms a stable 1:1:1 heterotrimer with PilN and PilO, and that formation of this complex protects its proteolytically sensitive, unstructured N terminus from degradation (161). Formation of such a complex in vivo is also thought to explain the observation that the lipidation site of PilP can be mutated without loss of function. The isolated β-domain of PilP is unable to form a stable complex with PilNO, likely because it is the region of PilP that interacts with the secretin PilQ (14, 161).
The last member of the proposed alignment subcomplex is FimV, a large (97 kDa) protein containing a periplasmic domain with a peptidoglycan-binding LysM motif connected via a single transmembrane segment to a highly acidic cytoplasmic domain containing predicted tetrapeptide repeat protein-protein interaction motifs (148). The peptidoglycan-binding function of FimV is required for correct formation of the PilQ secretin, as mutants deleted of the LysM motif have significantly fewer secretins and impaired motility (166). The protein may enable the massive secretin to pass through the covalently closed peptidoglycan layer and/or anchor the assembly system in the cell wall to brace it against the forces generated by pilus retraction (147). The function of the cytoplasmic domain of FimV is currently unknown, but it may participate in regulating pilus biogenesis (56) (see below).

The final subcomplex is, of course, the pilus itself. It is the most dynamic of the four, as it is repeatedly assembled and disassembled. It is composed of the major subunit PilA (130), as well as the minor (based on abundance) pilins FimU, PilVWX, and PilE (5, 6). Each *P. aeruginosa* strain expresses one of five PilA alleles, two of which are glycosylated, and one of two sets of minor pilins (32, 59, 94, 165). The major pilins can be exchanged between strains and function normally if accompanied by their cognate accessory genes that are linked to the pilin loci (9) (Figure 5); the exception is the group IV pilins, which also require their unique glycans biosynthetic machinery (68). The two sets of minor pilins are also interchangeable, with the exception of the PilX orthologs, which do not function in heterologous backgrounds (59).

Although the majority of the pilus fiber consists of PilA, the minor components are also present in surface-exposed pili (58, 174). Both major and minor pilins share the highly conserved N-terminal domain characteristic of T4P and T2S subunits, but their periplasmic C termini are divergent. Similar to PilA, minor pilins are processed by PilD, supporting the contention that they are assembled into the pilus (58, 174). The hydrophobic N termini of pilins act as transmembrane domains prior to assembly and as protein-protein interaction domains in the assembled fiber (43). Evidence from the T2SS suggests that the minor subunits form a complex that primes the subsequent assembly of major subunits below it, thereby forming the tip of a growing fiber (36, 52, 92). By analogy, the T4P minor pilins may form a similar initiation complex that is subsequently displayed at the distal ends of pili, though confirmation requires further studies. Immunogold microscopy showed infrequent labeling along pilus fibers, but it was not possible to distinguish whether the proteins were incorporated along the length of the pilus or present at the tips of bundled fibers of different lengths (58).

A large (~125 kDa) nonpilin protein called PilY1 is encoded within and coordinately regulated with the minor pilin operon (5, 26). PilY1 is important for pilus function, although its exact role is controversial (70, 93, 123). Its *Neisseria* homolog, PilC, is proposed to be a pilus tip adhesin, but to date PilY1 has yet to be convincingly associated with T4P or their tips. According to data from some studies (93, 123), PilY1 appears to have both direct and indirect regulatory effects on pilus function, including calcium-dependent control of pilus retraction. The question of whether PilA, minor pilins, or PilY1 is present at the tips of *P. aeruginosa* pili is a fundamental one, because the biochemical nature of the adhesin is likely to dictate the range and affinities of possible surface interactions. Although PilA was reported to be the T4P adhesin in *P. aeruginosa* (78), the contribution of other proteins must be examined to clarify their role.

How Does Twitching Happen?

In simplified terms, twitching is thought to result from removal of pilin subunits from the base of a previously assembled pilus fiber that is attached to a surface via its tip. Because both the pilus tip and the assembly system are fixed, shortening the pilus by disassembly pulls it increasingly
taut. The forces thus generated eventually overcome the friction caused by interactions of the cell body with the surface, pulling the cell toward the adhered pilus tip. This scenario assumes that the contact between the cell body and the surface is the weakest link, so that movement happens before the pilus tip releases, the fiber breaks, or the pilus or assembly system tears free of the cell. The exact mechanisms by which the T4P system assembles and then disassembles a pilus fiber that is under tension are currently the subject of intense research, but biochemical, biophysical, structural, and molecular modeling studies have provided important clues that allow some speculation about the process.

The first stage is pilus assembly. According to current models, the minor pilins, oriented with their C termini in the periplasm, may form an initiation complex that, because of its size and shape, partly protrudes from and deforms the membrane, lowering the amount of energy necessary to extract the hydrophobic N termini (36). Molecular simulations suggest that both pilins and T2S pseudopilins adopt a slight tilt in the membrane (36, 99), which may further assist in their extraction. The low abundance of the minor pilins probably ensures that few initiation sites relative to the amount of major pilins are available for subsequent polymerization, so that each pilus is long enough to extend a useful distance from the cell. Changing the stoichiometry of minor subunits can cause pili of aberrant length to form (53, 58). Although priming by the minor pilins is important for efficient assembly, it is not essential; mutants lacking all the minor pilins still assemble surface pili that can be observed in a retraction-deficient background (59).

To further extract the initiation complex from the membrane by adding additional pilin subunits beneath it, mechanical energy generated by hydrolysis of ATP by PilB is thought to be necessary. Structural data suggest that monomers of both PilB and PilT ATPases have head and body domains connected by a flexible linker (143, 146). In the functional unit, a hexamer, the head of each subunit interacts with the body of the adjacent subunit (55) (Figure 6). Comparison of PilT crystal structures in unbound and nucleotide-bound states showed large domain movements of the head relative to the body around the flexible linker (116, 146) that are presumably translated to neighboring subunits and/or other interaction partners. How these motions in a cytoplasmic protein complex result in extraction of a pilin on the periplasmic side of the membrane remains unanswered.

There is evidence from both T2S and T4P systems that the polymerase interacts with the N-terminal cytoplasmic domain of the platform protein (8, 44, 139), although the stoichiometry of both the platform protein and the potential interaction remains murky. If the transmembrane regions of the platform protein simultaneously interact with the membrane-embedded hydrophobic N termini of pilins, a conformational change in the ATPase bound to the platform protein’s cytoplasmic domain could shove it, its adjacent transmembrane domain(s), and an associated pilin subunit upward. The distance that a pilin needs to be pushed is ~1 nm, estimated by fiber structural studies (43) and molecular simulations (99) to be far enough to allow it to establish stable interactions with the preceding subunit. This proposed mechanism is reminiscent of an upside-down version of the process hypothesized for transmission of signals across the membrane by chemotaxis receptor proteins. In that case, binding of chemical ligands to the periplasmic domain of chemoreceptors is thought to cause conformational changes that induce piston-like downward movements of transmembrane helices, generating corresponding movements in their cytoplasmic domains (125, 131).

As the assembly system pushes pilins out of the membrane, subunit-subunit interactions would stabilize them in the assembled fiber. A highly conserved, negatively charged Glu residue at +5 of the mature subunit forms a critical salt bridge with the methylated N terminus of the previous subunit, ensuring the correct registration of one pilin relative to the next (43). Only PilX and related minor (pseudo)pilins lack this key residue (5), which may reflect a role as one of the
first proteins to move upward during initiation complex formation. A network of hydrophobic interactions between the pilins’ N termini, as well as additional interactions between the loop regions of the C-terminal domains, provides the pilus with its impressive ability to withstand large forces generated upon retraction. The mechanism of pilus length control is unknown but is probably related to the number of available pilins per initiation site (58) and to the frequency of disassembly relative to assembly.

The next stage of twitching is tethering of the pilus tip (or possibly greater portions of the fiber) to a surface (97, 155). This step is poorly characterized, in terms of both the specific biochemical nature of the pilus tip (above) and how an ultimately reversible bond with the surface that is firm enough to withstand retraction forces is achieved. Early studies of PilA function suggested that a disulfide loop located at the C terminus of the protein was the adhesive portion (176), using main chain atoms to bind to receptors on epithelial cells (10). Peptides corresponding to that portion of the protein were also reported to block subsequent pilus-mediated adherence to abiotic surfaces (60). However, these studies did not consider the presence of other components that might modulate attachment. This is an area of T4P function that requires more attention to better explain the amazing binding capacity of these fibers.

The final stage of twitching is pilus retraction, which involves switching from fiber polymerization to depolymerization in response to signals that are not yet understood. The way in which the pilus is reeled into the cell at the blistering pace of $\sim 10^3$ subunits per second (155) while under tension remains a mystery. Although the PilT ATPase is required, exactly how it promotes pilin depolymerization is still unknown. Logically, one might imagine that it could use a strategy similar to that proposed for PilB, but in the opposite direction. Rather than pushing pilins from the membrane upward via interactions with PilC, it could pull them from the pilus downward. The two large cytoplasmic domains of the PilC platform protein have $\sim 34\%$ sequence identity in P. aeruginosa and potentially a similar $\alpha$-helical bundle structure (83). If the N-terminal cytoplasmic domain interacts with PilB, the C-terminal domain might similarly interact with PilT. This proposed interaction is supported by a single piece of evidence showing that overexpression of this portion of the EPEC T4bP platform protein in trans mimics a retraction-deficient phenotype, potentially by titrating the retraction ATPase away from the system (44, 115). Alternatively, PilT could displace PilB in binding to the N-terminal cytoplasmic domain; however, there is currently no evidence for this interaction. Another possibility is that PilT interacts directly with the subunits (116), although how such an interaction could occur across the membrane to remove them from the pilus is not clear.

P. aeruginosa has two paralogous retraction ATPases, PilT and PilU (170, 172), that are not functionally interchangeable. Localization studies showed that whereas PilB and PilT are bipolar, PilU is unipolar (34), suggesting a possible role in regulating pilus function (below) at one pole versus the other. pilU mutants are piliated but, unlike pilT mutants, remain susceptible to killing by pilus-specific bacteriophages (170, 172). This phenotype is consistent with a small amount of residual motility (66), implying that PilT continues to function, albeit inefficiently, in the pilU background. For now, the way in which PilU modulates PilT function is unknown.

WHERE ARE THEY GOING? REGULATION OF TWTITCHING MOTILITY

Twitching allows bacteria to travel, but where are they going and how do they navigate? In P. aeruginosa, twitching is controlled by a number of regulatory systems that sense external signals—most of which are unknown—and transduce them to modulate pilus extension and retraction. Both physical (e.g., viscosity) and chemical (e.g., phospholipids, iron) signals that influence twitching...
have been identified (16, 22, 85, 154), as have several regulatory proteins and protein relays (4, 18, 19, 46, 75, 84, 159, 169). Supplemental Figure 1 summarizes many of the regulatory inputs that control twitching, although a comprehensive understanding of the players and the extent and nature of their interconnectedness continues to evolve. The contributions of a subset of regulatory factors are discussed below.

**Regulation of Pilin and Minor Pilin Expression by Two-Component Systems**

Among the simplest mechanisms of regulation is expression (or not) of the major pilin subunit, PilA. In *P. aeruginosa*, as in many other T4aP-expressing bacteria, transcription of the *pilA* gene is controlled by the alternate sigma factor, RpoN (σ54), and a two-component system, PilR-PilS (114, 159). PilS, the sensor kinase, has six membrane-spanning domains and is localized to both poles (27). Autophosphorylation of PilS and subsequent phosphorylation of its response regulator, PilR, upregulate *pilA* transcription. The exact signal sensed by PilS has not yet been determined but is potentially PilA itself. Bertrand et al. (22) showed that alteration of inner membrane PilA pools, either by inactivation of genes encoding PilA or by the ATPases PilB or PilT, affected *pilA* transcription. In a *pilB* mutant, where unassembled pilins accumulate in the inner membrane, *pilA* transcription was reduced. In *pilT* mutants, where pilin pools were depleted because of unopposed assembly in the absence of retraction, *pilA* transcription was increased. The ability of PilS to sense and respond to PilA levels in the inner membrane could occur directly, via protein-protein interactions between the highly conserved, membrane-embedded hydrophobic N terminus of PilA and the transmembrane segments of PilS. This hypothesis would account for the ability of PilS—which is identical among unrelated strains of *P. aeruginosa* (59)—to control the levels of pilins whose C-terminal sequences are divergent. Older studies of *M. xanthus* pilin expression (177) support this idea, as specific point mutations in the conserved N terminus of the pilin did not affect its stability but led to dysregulation of expression.

The minor pilin operon is under control of a separate two-component regulatory system, AlgR-FimS (20, 169). Although other genes are part of the AlgR regulon, its effects on pilus biogenesis are limited to control of minor pilin expression. Provision of the operon (but not individual open reading frames) in trans in an *algR* background is sufficient to restore pilus biogenesis (102). The alternate sigma factor AlgU (σ22) controlling alginate synthesis also modulates twitching motility (169), although its effects appear to be indirect. AlgU controls the expression of the lectin LecB (17), required for PilJ expression (158), and PilJ controls pilus biogenesis (below).

**cAMP-Dependent Regulation of T4aP Biogenesis**

Whereas regulation of pilin transcription is relatively straightforward, regulation of pilus assembly is more complex owing to the large number of components involved. Early in the investigation of *P. aeruginosa* T4aP biogenesis, a putative pilus-specific chemotaxis system (Pil-Chp) composed of components that share sequence similarity with those of the flagellar chemotaxis system of *E. coli* was identified (45–47, 171). The canonical flagellar system consists of membrane-bound methyl-accepting chemotaxis proteins (MCPs) that sense environmental stimuli and undergo a conformational change transmitted via an adaptor protein, CheW, to a histidine kinase, CheA. After autophosphorylation, CheA phosphorylates CheY, which binds to the flagellar switch complex to induce conformational changes that ultimately reverse the direction of flagellar motor rotation (133, 145). The extent of CheY phosphorylation is controlled by CheZ, and sensitivity of the MCPs to signals is controlled by their methylation and demethylation via CheR and CheB, respectively (142). Although the Pil-Chp system has identifiable homologs of many Che proteins,
the extension-retraction mechanism of the pilus motor is clearly different from that of the rotary flagellum motor.

The Pil-Chp system has a single MCP, PilJ, localized to both poles of the cell (50). PilI is a CheW homolog and ChpA (PilL) is a more complicated version of CheA, with a combination of nine His, Ser, and Thr phosphotransfer domains and one C-terminal CheY-like domain (171). There are two CheY-like regulators, PilG and PilH, but no CheZ homolog. Fulcher and colleagues (56) proposed that in the absence of a phosphatase, PilH may act as a phosphate sink to control the extent of PilG phosphorylation. PilK and ChpB encode homologs of CheR and CheB, respectively. ChpC is predicted to be a second CheW homolog that may link other MCPs to ChpA (171). Similar chemotaxis systems can be identified in many other soil-, water-, and plant-associated bacteria that have T4aP, such as *Myxococcus, Xylella, Xanthomonas*, and *Ralstonia*, but not all T4aP-expressing bacteria encode such a system. Pathogens such as *Neisseria, Legionella*, and *Vibrio* lack Pil-Chp systems.

Recent analyses of *P. aeruginosa* pil-chp mutants confirmed that pilG, pilI, pilJ, and chpA are involved in pilus assembly, as mutants had little or no surface pili despite having normal levels of intracellular PilA (22, 56). Similarly, a lecB mutant, which has a substantial reduction in PilJ levels, does not assemble pili but has normal PilA levels (158). In contrast, pilH, pilK, chpB, and chpC mutants continued to twitch, although surface pilus levels were slightly altered in some strains; in particular, pilH and chpB mutants were reported to be hyperpiliated, suggesting their inactivation has effects on retraction. From these and other data, Bertrand et al. (22) proposed that PilG may control PilB activity and thus assembly, and PilH might affect PilT activity and therefore retraction. Although this is a simple and attractive hypothesis, further discoveries suggested that the regulatory picture is more complex (56, 76).

The expression of many *P. aeruginosa* virulence factors—including T4aP—is controlled in part by a cyclic AMP (cAMP)-binding protein called Vfr (virulence factor regulator) (167). cAMP is synthesized by two adenylate cyclases, CyaA and CyaB, with CyaB being the major contributor to cellular cAMP pools (56). While searching for factors that affected CyaB activity, Fulcher et al. (56) isolated mutants in the pil-chp genes, as well as others implicated in pilus biogenesis or function (*pilA, pilB, fimL, fimV, vfr*). Mutations that affected pilus biogenesis (*pilG, pilI, pilJ, chpA, fimL, fimV*) reduced cellular cAMP levels—similar to those of a cyaAB double mutant—even though CyaB protein levels were unaffected. Similarly, FimL appears to positively regulate CyaB activity, as mutants had normal amounts of CyaB but low cAMP levels (76). In contrast, pilH, pilK, and chpB mutants had high levels of cAMP and greater than wild-type levels of surface pili. From these data, PilG and PilH were proposed to modulate CyaB activity and thus the expression of Vfr-dependent proteins, including PilMNOPQ (81), among others. However, it was also apparent that the Pil-Chp system had cAMP-independent effects on pilus function, as supplying a cyaAB-pilG triple mutant with exogenous cAMP restored pilus biogenesis but not motility (56).

FimV appears to have dual roles in pilus biogenesis, consistent with its two-domain structure. Its periplasmic N terminus binds peptidoglycan and promotes PilQ secretion formation in the outer membrane (166), and its cytoplasmic C terminus may positively regulate CyaB function (56). The decreased levels of PilMNOP previously reported in fimV mutants (166) may be related to low cAMP levels and thus Vfr-dependent transcription (81). FimV also affected function of the T2SS under specific growth conditions (112), also supporting a broader regulatory function.

**Cyclic-di-GMP Signaling and Twitching Motility**

Cyclic-di-GMP (cdG) is another key secondary messenger in bacteria that has received considerable attention because of its role in promoting biofilm formation (40, 71) and other
phenotypes associated with chronic *P. aeruginosa* infections (103). Proteins involved in cdG metabolism have characteristic motifs GGDEF (found in diguanylate cyclases) and/or EAL (found in phosphodiesterases). In some proteins, degenerate forms of these motifs simply bind the molecule. In addition, a host of other cdG-binding motifs continue to be identified, including allosteric regulatory sites controlling enzymatic activity and RNA riboswitches controlling expression.

Two cdG-related proteins required for T4P biogenesis have been identified, PilZ and FimX. PilZ mutants express normal amounts of pilins but do not assemble pili and are resistant to pilus-specific phage (4). When identified, PilZ had a novel sequence that was subsequently identified as a archetypal cdG-binding adaptor domain, found in a large and diverse family of proteins (7). Ironically, although binding of cdG by many members of the PilZ domain family has since been demonstrated, PilZ itself (PA2960) does not bind the molecule (109). Structural studies (101) revealed that PilZ and its orthologs in other T4P systems have critical structural differences at the N terminus where members of the larger PilZ domain family typically bind cdG. Subsequent studies in *Xanthomonas* spp. showed that PilZ interacts with both PilB, the pilin polymerase, and the nonfunctional EAL domain of FimX, a cdG-binding protein (64). This work suggested that PilZ together with FimX might regulate PilB function in a cdG-dependent manner.

*P. aeruginosa* FimX is a unipolar signaling protein containing a CheY-like REC domain, a PAS sensor domain, and degenerate GGDEF (GDSIF) and EAL (EVL) domains (75, 84), implying that it senses and integrates environmental signals relevant to twitching to influence pilus function. Mutants lacking *fimX* have reduced twitching and respond only weakly to specific environmental cues (e.g., the presence of mucin) that markedly stimulate twitching of wild-type cells. The noncatalytic C-terminal EVL domain binds cdG with high affinity (\( K_d \sim 100 \text{ nM} \)), causing a long-range (\( \sim 7 \text{ nm} \)) conformational change to be transduced to the N-terminal REC domain responsible for unipolar localization (118, 140). Deletion of the GDSIF or EVL motifs prevents localization and function (84), suggesting that integration of extracellular cues with intracellular levels of cdG is required to modulate twitching. *P. aeruginosa* has multiple diguanylate cyclases and phosphodiesterases that can modulate cdG levels. Many are linked to other signaling pathways, so it will be interesting to determine which ones connect to FimX, and how the circuits connecting cdG- and cAMP-based control of twitching are organized.

**LOOKING FORWARD**

The field of twitching motility has seen tremendous progress in many areas over the past decade. The discovery of the many ways beyond simple crawling motility in which T4P contribute to bacterial movement and biofilm development was made possible by development of elegant microscopy and biophysical techniques coupled with sophisticated particle-tracking algorithms. Other notable achievements include an increasingly detailed picture of the molecular structure of the T4P machinery and its potential mechanism. The rapid characterization of many of the previously identified genes that were implicated in T4P function but whose roles were enigmatic has been particularly interesting. Many of these mysterious *pil* and *fim* genes have turned out to be key regulators of pilus biogenesis or pilus function, and it will be exciting to add more pieces to this incomplete jigsaw puzzle.

Topics needing further attention include the similarities and differences between T4a, T4b, and Tad pili; their assembly systems; and their regulatory components. Most of our structural information comes from the T4a system, and with a single exception (179), there is almost nothing known about the structure of the Tad pilus and its machinery. A more complete understanding of how these systems evolved and how they function would be very useful. In particular, identification
of key targets may aid in the development of type IV pilicides that could be used in antivirulence strategies. This approach has been used successfully for other types of bacterial fimbriae involved in pathogenesis (136). The stoichiometry and spatial organization of the components within the T4P complexes and the manner in which the systems are organized in the cell envelope, including the peptidoglycan layer, are poorly defined. Are they built-in during cell division when new poles are formed, or retrofitted afterward? Do components of the assembly systems form stable complexes, or are specific subcomplexes (in addition to the pilus) dynamic? These areas and more await revelation in the next 10 years.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

I am very grateful to the past and present members of my laboratory, and to my long-term collaborator Lynne Howell and her trainees, for their hard work and interesting ideas about all things pili. To those in the field, I’m sorry that space limitations made it impossible to cover all aspects with equal attention. Work in the Burrows laboratory on T4P is generously funded by the Canadian Institutes of Health Research.

LITERATURE CITED


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