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Therapeutic Approaches Targeting the Assembly and Function of Chaperone-Usher Pili

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ABSTRACT The chaperone-usher (CU) pathway is a conserved secretion system dedicated to the assembly of a superfamily of virulence-associated surface structures by a wide range of Gram-negative bacteria. Pilus biogenesis by the CU pathway requires two specialized assembly components: a dedicated periplasmic chaperone and an integral outer membrane assembly and secretion platform termed the usher. The CU pathway assembles a variety of surface fibers, ranging from thin, flexible filaments to rigid, rod-like organelles. Pili typically act as adhesins and function as virulence factors that mediate contact with host cells and colonization of host tissues. Pilus-mediated adhesion is critical for early stages of infection, allowing bacteria to establish a foothold within the host. Pili are also involved in modulation of host cell signaling pathways, bacterial invasion into host cells, and biofilm formation. Pili are critical for initiating and sustaining infection and thus represent attractive targets for the development of antivirulence therapeutics. Such therapeutics offer a promising alternative to broad-spectrum antibiotics and provide a means to combat antibiotic resistance and treat infection while preserving the beneficial microbiota. A number of strategies have been taken to develop antipilus therapeutics, including vaccines against pilus proteins, competitive inhibitors of pilus-mediated adhesion, and small molecules that disrupt pilus biogenesis. Here we provide an overview of the function and assembly of CU pili and describe current efforts aimed at interfering with these critical virulence structures.

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INTRODUCTION

The chaperone-usher (CU) pathway is dedicated to the biogenesis of surface structures termed pili or fimbriae that play indispensable roles in the pathogenesis of a wide range of bacteria (1–4). Pili are hair-like fibers composed of multiple different subunit proteins. They are typically involved in adhesion, allowing bacteria to establish a foothold within the host. Following attachment, pili modulate host cell signaling pathways, promote or inhibit host cell invasion, and mediate bacterium-bacterium interactions leading to formation of community structures such as biofilms (5, 6). Gram-negative bacteria express multiple CU pili that contribute to their ability to colonize diverse environmental niches (1, 7–10). Pili thus function at the host-pathogen interface to both initiate and sustain infection and represent attractive therapeutic targets.

PILUS FUNCTION

The most extensively characterized CU pili are type 1 pili, found in members of the *Enterobacteriaceae*, and P pili, found in uropathogenic *Escherichia coli* (UPEC). Both pili are key virulence factors for UPEC colonization of the urinary tract and the establishment of urinary tract infections (UTI) (Fig. 1). Type 1 pili bind to mannosylated proteins in the bladder, leading to cystitis, and P pili bind to di-galactose-containing moieties in kidney glycolipids, leading to pyelonephritis (11–13). Bacterial binding via type 1 pili also activates host cell pathways that lead to actin cytoskeletal rearrangements and subsequent bacterial invasion into the host cells via a zipper-like mechanism (14, 15). Type 1 pili contribute to the formation of extracellular biofilms (16), as well as intracellular biofilm-like communities (IBCs) by UPEC during bladder infection (Fig. 1) (17). Bacteria within these IBCs are protected from antibiotics and immune surveillance (18, 19).

Type 1 and P pili expressed by UPEC are considered classical pili, which are heteropolymers of different protein subunits that form rigid, helical rods. Similarly, enterotoxigenic *E. coli* (ETEC) employs a large group of rigid pili, termed colonization factor antigen (CFA) or coli surface antigen (CS) pili, to adhere to the small intestine, facilitating toxin delivery into the gut lumen (20). Another group of pili assembled by the CU pathway comprises thin, flexible fibers that in some cases form amorphous, capsular-like or "afimbrial" structures (3). Examples of these are the Afa/Dr pili (21–23), expressed by various pathogenic *E. coli* strains, and the F1 capsular antigen of *Yersinia pestis* (24, 25), which forms a dense coating around the bacteria and is involved in preventing uptake by macrophages (Fig. 1) (25, 26).

CU pili are remarkably adapted to colonization of specific environmental niches. To mediate colonization of the urinary tract, type 1 pili must be able to withstand the shear forces generated by the flow of urine. The FimH adhesin utilizes a catch bond mechanism to switch between low- and high-affinity binding conformations, facilitating migration (rolling) and receptor sampling in the

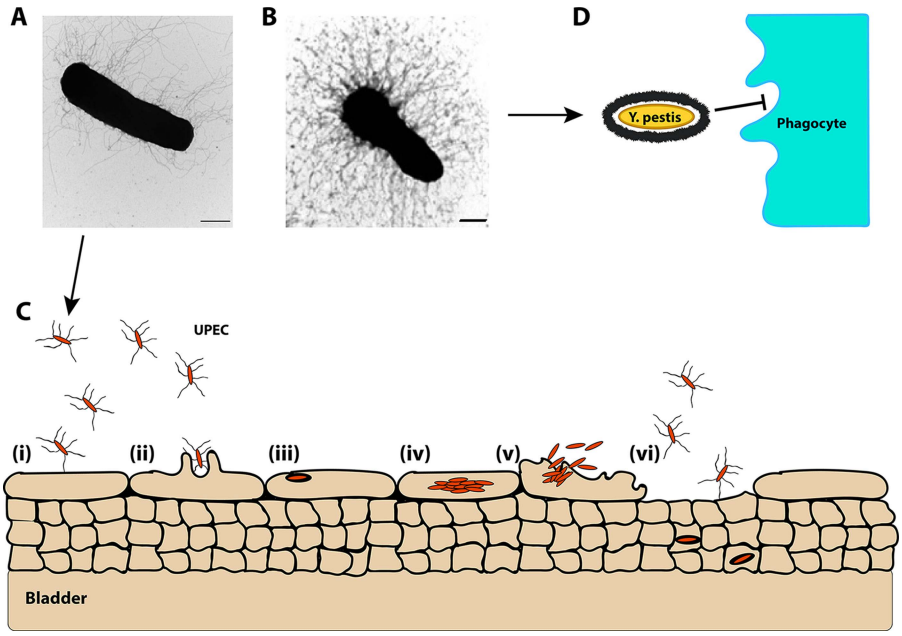

Figure 1 Ultrastructure and function of CU pili. Electron micrographs of *E. coli* expressing type 1 pili **(A)** and *Y. pestis* expressing F1 capsule **(B)**. Scale bars = 500 nm. **(C)** Cartoon for pilus-mediated bacterial interactions in the bladder. (i) Type 1-piliated UPEC binds to superficial umbrella cells that line the lumen of the bladder. (ii) Pilus-receptor interactions induce a signaling cascade that promotes internalization of adherent bacteria via a membrane zippering mechanism. (iii) Within bladder epithelial cells, UPEC are trafficked to membrane-bound, acidic compartments similar to lysosomes. (iv) In the superficial umbrella cells, UPEC break into the cytosol and rapidly multiply, forming intracellular biofilm-like communities. (v) Bladder cells containing large numbers of UPEC exfoliate, providing a mechanism for bacterial clearance by the flow of urine. (vi) This, however, leaves the underlying layers of immature bladder epithelial cells exposed. UPEC can invade these immature urothelial cells and persist in a quiescent stage in late endosome-like compartments, avoiding detection by immunosurveillance mechanisms. **(D)** The *Y. pestis* F1 capsule plays an antiphagocytic role by preventing opsonizing antibodies from binding to the bacterial surface, blocking Fc receptor phagocytosis. More generally, expression of the F1 capsule can mask bacterial adhesins and other surface structures, preventing interactions that lead to internalization into host cells.

absence of urinary flow and attachment (sticking) during periods of turbulence (27–29). The helical pilus rod exhibits properties of compliance and flexibility, which is also important for resistance to shear forces and allows bacteria to regain proximity to host cells after exposure to turbulence (30–32).

PILUS ASSEMBLY

The CU pathway harnesses protein-protein interactions to drive pilus fiber assembly and secretion in the absence of an external energy source such as ATP, which is not available in the bacterial periplasm (33, 34). Newly synthesized pilus subunits in the cytoplasm contain an N-terminal signal sequence that directs them to the SecYEG translocon in the inner membrane for translocation into the periplasm (Fig. 2). In the periplasm, the signal sequence is cleaved and the subunits undergo disulfide bond formation in a process catalyzed by the oxidoreductase DsbA (33, 35). The subunits then form binary complexes with chaperone proteins (FimC for type 1 pili and PapD for P pili). The chaperone recognizes only unfolded subunits that have already undergone disulfide bond formation. This serves an important quality control role, ensuring that only oxidized, mechanically stable subunits are incorporated into the pilus (36–38). The chaperone donates a β-strand to complete the immunoglobulin-like fold of the subunits in a mechanism termed donor strand complementation (DSC) (39, 40). This process allows subunit folding and inhibits premature subunit-subunit interactions. In the absence of the chaperone, subunits misfold, aggregate, and are degraded by the DegP periplasmic protease (41, 42).

Periplasmic chaperone-subunit complexes interact with the outer membrane (OM) usher (FimD for type 1 pili and PapC for P pili) (Fig. 2), which catalyzes the exchange of chaperone-subunit for subunit-subunit interactions in a process termed donor strand exchange (DSE) (43–47). In DSE, the N-terminal extension of an incoming pilus subunit displaces the β-strand donated by the chaperone to release the chaperone and form a subunit-subunit interaction. This interaction is energetically favorable and initiates at a binding pocket on the subunit, termed the P5 pocket, which is left vacant by the chaperone donor strand (43–46). The correct ordering of subunits in the pilus fiber is determined by the differential affinities of chaperone-subunit complexes for the usher and the rate of DSE between different subunit-subunit pairs, as well as the periplasmic concentrations of different subunits (47–51). The usher thus promotes ordered polymerization of the pilus fiber and provides the channel for secretion of the pilus fiber to the cell surface.

The usher comprises a 24-stranded β-barrel channel domain, a plug domain that serves as a channel gate, an N-terminal periplasmic domain (NTD), and two C-terminal domains (CTD1 and CTD2) (52–55). In the resting (*apo*) usher, the plug domain occludes the channel pore and masks the usher C domains (56, 57). In the type 1 pilus system, the usher is activated by binding of a FimC-FimH chaperone-adhesin complex to the usher NTD (52). FimC-FimH binding results in plug expulsion from the lumen of the usher channel, which also frees the CTDs (55, 57) (Fig. 2). FimC-FimH is then delivered from the usher NTD to the CTDs, in a handover process likely driven by differential affinity and direct domain-domain interactions (57–60). This frees the usher NTD for recruiting the next chaperone-subunit complex (FimC-FimG for type 1 pili) from the periplasm. The newly recruited complex bound to the usher NTD is oriented perfectly to undergo DSE with the previously recruited complex bound at the usher CTDs (55) (Fig. 2). This displaces the chaperone from the subunit bound at the CTDs, forming the first link in the pilus fiber. The newly incorporated chaperone-subunit is then handed over from the usher NTD to the CTDs to reset the system for a new round of subunit recruitment and incorporation, which continues concomitantly with translocation of the nascent pilus fiber through the usher channel to the cell surface. The pilus fiber is thus assembled and secreted in a top-down manner, with the pilus rod adopting its final helical quaternary structure upon exiting the usher pore. The helical rod is stabilized by extensive polar interactions between the pilus rod subunits, providing a remarkable level of flexibility that is important for resistance to shear stress. Shear stress can disrupt these polar interactions, linearizing the pilus rod without breaking the strong hydrophobic interactions that mediate subunit polymerization (61–63). Each of these steps along the CU assembly pathway offers targets for the development of therapeutic inhibitors.

PILUS-DIRECTED THERAPEUTIC APPROACHES

The ever-increasing rate of antibiotic resistance among pathogenic bacteria is necessitating a hard look into alternative methods for treatment (64, 65). Antivirulence therapeutics that specifically target pilus function or

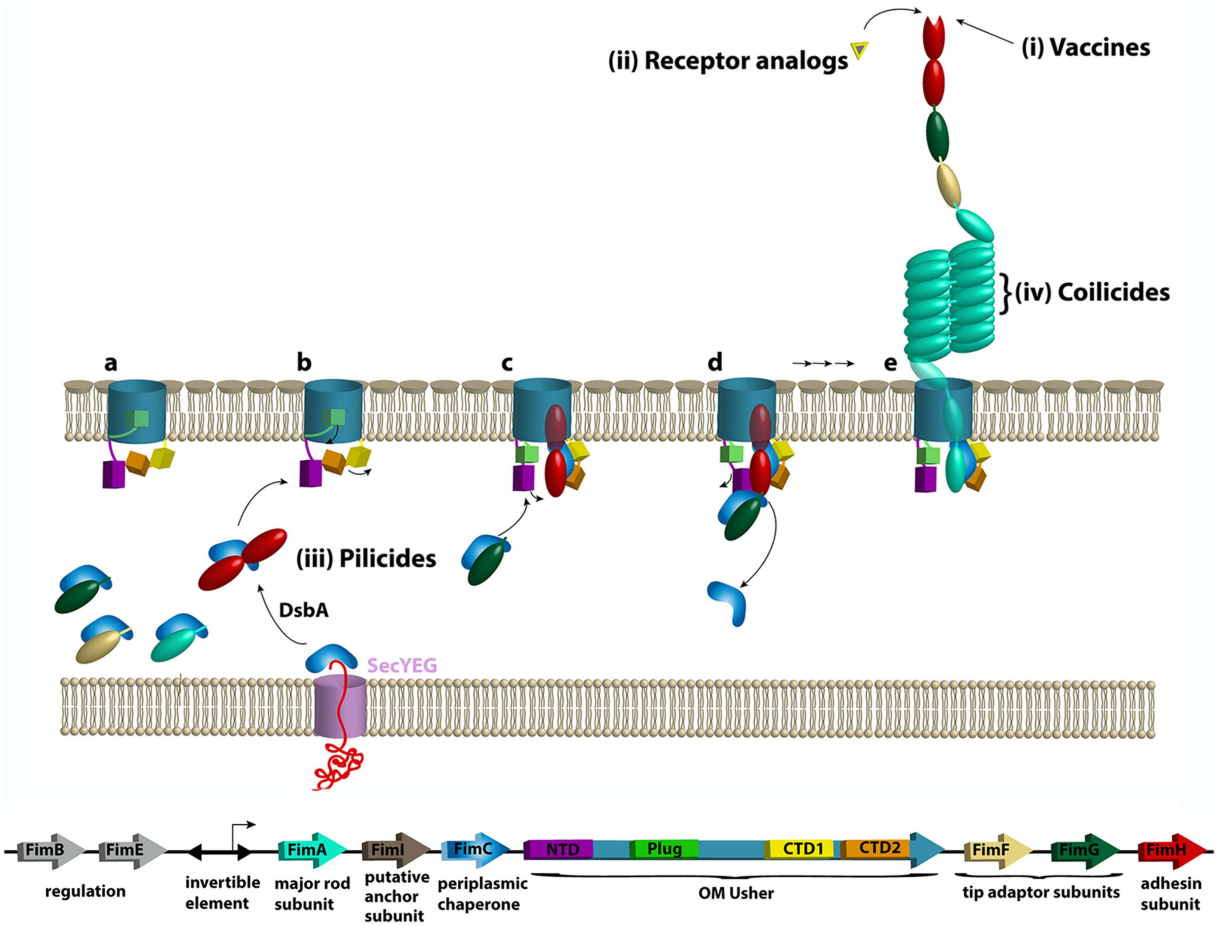

Figure 2 CU pilus assembly pathway and targets for therapeutic intervention. The *fim* gene cluster coding for type 1 pili, along with names and functions of encoded proteins, is shown at the bottom. Upon entering the periplasm via the SecYEG general secretory machinery, nascent pilus subunits form binary complexes with the pilus chaperone (FimC), which facilitates subunit folding by DSC, completing the Ig fold of the subunit's pilin domain. The adhesin subunit (FimH, red) is depicted with an additional N-terminal lectin domain, which contains the receptor-binding site. Chaperone-subunit complexes then interact with the OM usher (FimD), which comprises a β-barrel channel domain, a plug domain, an N-terminal periplasmic domain (NTD), and two C terminal domains (CTD1 and CTD2). (a) In the resting usher, the plug domain occludes the channel pore and masks the CTDs. (b and c) The usher is activated by binding of a FimC-FimH chaperone-adhesin complex to the usher NTD. This results in displacement of the plug from the channel and handoff of FimC-FimH to the usher CTDs, freeing the NTD to recruit the next chaperone-subunit complex (FimC-FimG). (d) The newly recruited complex bound to the NTD is oriented perfectly to undergo DSE with the previously recruited complex bound to the CTDs, forming the first link in the pilus fiber. The newly incorporated chaperone-subunit is then handed over from the NTD to the CTDs. (e) Repeated rounds of this process result in assembly and secretion of the pilus fiber. Different steps along this pathway are targets for antipilus therapeutics. (i) Vaccination using a full-length or truncated adhesin subunit inhibits pilus-mediated bacterial adhesion and pathogenesis. (ii) Small-molecule receptor analogs occupy the pilus adhesin binding site, preventing pili from adhering to host receptors. (iii) Pilicides inhibit pilus assembly via different mechanisms, such as interfering with chaperone-subunit or subunit-subunit interactions, interfering with binding of chaperone-subunit complexes to the usher, or inhibiting proper folding of the usher in the bacterial OM. (iv) Coilicides inhibit uncoiling and recoiling of the pilus rod, thus impairing resilience of the fibers during fluid flow.

assembly represent one such alternative approach to traditional antibiotics. In contrast to traditional antibiotics that nonspecifically interfere with essential biological processes, antivirulence therapeutics disrupt systems only required for bacterial pathogens to cause disease within the host, thus limiting detrimental side effects on commensal bacteria and the selective pressure that leads to antibiotic resistance (66–69).

The indispensable roles that CU pili play in bacterial pathogenesis make them attractive targets for directed therapeutic intervention. A number of approaches have been taken to develop antipilus therapeutics, including vaccines against pilus proteins, inhibitors of pilus-mediated adhesion, and small molecules that disrupt pilus biogenesis (Fig. 2). CU pili are prevalent among the *Enterobacteriaceae* and their structure, function, and mechanism

of assembly are conserved, suggesting that pilus-targeting therapeutics may have activity against a broad range of bacterial pathogens.

Vaccination Strategies

Because of their important roles in bacterial virulence, pili have received considerable attention in vaccine development programs. Vaccination with whole pili has proven unsuccessful, mainly due to factors such as phase-variable expression and antigenic variation (70, 71). Moreover, in the case of monoadhesive pili such as type 1 and P pili, which have a single distal adhesin subunit, this approach may bias the immune system towards structural pilus subunits that are present in much higher copy than the adhesin, thus failing to inhibit pilus function (6). In contrast, vaccination with the adhesin subunit FimH (type 1 pili) or PapG (P pili) confers substantial protection against UPEC in both murine and primate infection models, without affecting the commensal *E. coli* in the gut (72–76). Vaccination with a truncated form of the mannose-resistant *Proteus*-like fimbriae (MR/P) tip adhesin MrpH, fused to FliC from *Salmonella enterica* serovar Typhimurium as an adjuvant, was also found to confer protection against UTI caused by *Proteus mirabilis* (77). Moreover, in a recent human clinical trial, oral administration of antibodies raised against the colonization factor I (CFA/I) pilus tip adhesin CfaE conferred substantial protection against ETEC colonization (78).

The catch-bond behavior of pilus adhesins poses some challenges to vaccination. In some cases, antibody binding to FimH stabilizes its high-affinity state and thereby enhances rather than inhibits binding of the adhesin to its receptor (79). Moreover, by shifting from the high- to the low-affinity state, FimH may shed bound antibodies (79). Novel approaches have been taken to overcome these issues. For example, a new type of antibody that binds to a single loop within the binding pocket of FimH can displace bound ligand. This parasteric antibody is potent not only in inhibiting but also in reversing bacterial adhesion, dissolving surface-adherent biofilms and conferring protection against cystitis in mice (80).

Small-Molecule Receptor Analogs

For type 1 pili, soluble receptor analogs, termed mannosides, are being developed as antiadhesives by occupying the FimH receptor binding site. Mouse model studies have shown that these compounds can prophylactically prevent bacterial bladder colonization, with efficacy against established UTI as well as catheter-associated UTI (81–84). In a recent study, mannosides were found to selectively deplete intestinal UPEC reservoirs without altering the gut microbiota, which may have implications for reducing the rate of recurrent UTIs (85). Furthermore, by shifting the UPEC niche primarily to the extracellular milieu, mannosides may exhibit synergy with traditional antibiotics (86).

A variety of approaches are being taken to develop mannosides with improved or novel properties. FimH antagonist efficacy has traditionally been evaluated using a truncated FimH construct locked in a single conformation. New approaches taking into consideration the dynamic nature of FimH binding have led to the development of biphenyl mannosides that have excellent affinities for all physiologically relevant FimH conformations and exhibit increased potency compared to conventional FimH antagonists (87). Thiomannosides are reported to have improved metabolic stability and oral bioavailability (88). Galabiose-based soluble receptor analogs are also being developed to target P pilus adhesion (89). A similar strategy is being employed to develop receptor-mimicking galactosides that target the F9 pilus adhesin FmlH. Lead FmlH antagonists significantly reduce bacterial burdens in the bladders and kidneys of infected mice (90). Thiazolylmannosides and heptylmannoside-based glycocompounds, a new class of FimH antagonists with greater stability at low pH, have shown efficacy against adherent-invasive *E. coli*, which plays a key role in the gut inflammation of patients with Crohn's disease (91–94). Finally, multivalent inhibitors that function as potent anti-adhesives by cross-linking bacteria have also been developed by coupling FimH antagonists on synthetic scaffolds (95–97).

Small-Molecule Pilicides

Another class of small-molecule CU pilus inhibitors, known as pilicides, inhibit the pilus assembly and secretion process. The original pilicides consist of molecules with a 2-pyridine scaffold (98, 99). These molecules bind to the periplasmic chaperone and interfere with chaperone-subunit interactions or the binding of chaperone-subunit complexes to the usher. These compounds have demonstrated efficacy against pilus-mediated adhesion and biofilm formation (99–101). New synthetic approaches have resulted in the development of new classes of pilicides with improved potency (102, 103). In a recent study, pilicide ec240 was found to disrupt assembly of type 1, P, and S pili, as well as flagellar motility. In addition to interfering

with pilus assembly, ec240 induces *fimS*-mediated phase off variation, downregulating type 1 pilus gene expression. Treatment of UPEC with ec240 reduced biofilm formation and bacterial colonization in the mouse UTI model (104).

Computational screening for compounds with complementarity to the FimH P5 binding pocket led to identification of the small-molecule AL1, which inhibits pilus subunit polymerization by disrupting the DSE reaction between the FimH and FimG subunits (105). By disrupting type 1 pilus biogenesis, AL1 reduces biofilm formation and bacterial adhesion to human bladder epithelial cells. Another small-molecule compound, nitazoxanide (NTZ), was shown to inhibit biofilm formation by enteroaggregative *E. coli* by disrupting the assembly of AAF CU pili (106). Further analysis demonstrated that NTZ also inhibits type 1 and P pilus assembly via a novel mechanism of action, by interfering with proper folding of the usher protein in the OM (107).

Other Approaches

A novel approach to target bacterial adhesion is the use of coilicides, a recently developed class of antipilus inhibitors that act by impairing compliance of the CU pilus rod. In a proof-of-principle experiment, it was shown that purified PapD chaperone binds to uncoiled P pilus rods and prevents their recoiling, thus decreasing their ability to withstand shear forces caused by fluid flow (108). Similarly, polyclonal anti-PapA antibodies were found to reduce the elastic properties of P pili (109). Bivalent polyclonal antibodies have also been used to diminish the compliance of CFA/I and coli surface antigen 2 (CS2) pili, which play essential roles in ETEC pathogenesis. These antibodies, which recognize major pilin subunits, decrease pilus resilience during fluid flow by clamping together layers of the helical fiber or two individual pili, thereby increasing their stiffness and entangling them (110, 111). The salivary peptide histatin-5 was also found to bind to and stiffen CFA/I pili, inhibiting ETEC colonization in the gastrointestinal tract (112).

Another strategy that is being explored is the engineering of an avirulent asymptomatic bacteriuria strain, 83972, to synthesize a surface-located oligosaccharide P pilus receptor mimic. This strain can bind virulent P-piliated UPEC, impairing its adhesion to kidney epithelial cells (113, 114). In an alternate approach, a recombinant strain 83972 that expresses type 1 pili can interfere with urinary catheter biofilm formation by virulent enterococci (115).

CONCLUSIONS

Pili assembled by the CU pathway function as virulence factors for a range of Gram-negative pathogenic bacteria. Pili are attractive targets for therapeutic intervention, as they are required both for early stages of colonization in the host and maintenance of infection. Therapeutic agents that target CU pili such as vaccines, adhesin receptor analogs, and small molecules show promise in selectively disrupting host-pathogen interactions that are crucial for disease. Such agents offer alternatives to traditional antibiotics and a pathway forward to combat the rising threat of antibiotic resistance. Additional knowledge gained regarding the assembly, structure, and function of CU pili will provide new opportunities for the development of novel anti-infective therapeutics.

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