Pseudomonas aeruginosa virulence and therapy: Evolving translational strategies*

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Objective: **Although most reviews of *Pseudomonas aeruginosa* therapeutics focus on antibiotics currently in use or in the pipeline, we review evolving translational strategies aimed at using virulence factor antagonists as adjunctive therapies.**

Data Source: **Current literature regarding *P. aeruginosa* virulence determinants and approaches that target them, with an emphasis on type III secretion, quorum-sensing, biofilms, and flagella.**

Data Extraction and Synthesis: ***P. aeruginosa*** **remains one of the most important pathogens in nosocomial infections, with high associated morbidity and mortality. Its predilection to develop resistance to antibiotics and expression of multiple virulence factors contributes to the frequent ineffectiveness of current therapies. Among the many *P. aeruginosa* virulence determinants that impact infections, type III secretion, quorum sensing, biofilm formation, and flagella have been the focus on much recent investigation. Here we review how increased understanding of these important bacterial structures and processes has enabled the development of novel approaches to inhibit each. These promising translational strategies may lead to the development of adjunctive therapies capable of improving outcomes.**

Conclusions: **Adjuvant therapies directed against virulence factors have the potential to improve outcomes in *P. aeruginosa* infections. (Crit Care Med 2009; 37:1777–1786)**

KEY WORDS: *Pseudomonas aeruginosa*; virulence factors; type III secretion; quorum sensing; biofilms; flagella; adjunctive therapy

In intensive care units, *Pseudomonas aeruginosa* (PA) ranks among the top five organisms causing pulmonary, bloodstream, urinary tract, surgical site, and soft tissue infections (1). Current treatments, primarily antibiotics that kill or inhibit the growth of this bacterium (2), have been associated with unacceptably high rates of morbidity and mortality. The development of agents that antagonize virulence factors represents a novel and potentially fruitful approach to the treatment of severe infections caused by PA.

Any attempt to therapeutically target virulence determinants must build on a thorough understanding of host-pathogen interactions in PA infections (3). Interactions between PA virulence factors and the host immune response dictate the severity and type of infection. Depending on the environmental conditions and the immune status of the host, PA can be a quiescent colonizer, a cause of chronic infection, or a highly virulent invader during acute infections (3). For example, in the respiratory tract PA may cause fulminant and acute ventilator-associated pneumonia (VAP), be a colonizer in chronic obstructive pulmonary disease, or cause a chronic infection in cystic fibrosis (CF) patients, leading to slowly progressive deterioration of pulmonary function (3, 4). Bacterial surface factors, such as flagella, pili, and lipopolysaccharide, as well as active processes, such as the secretion of toxins, biofilm formation, and quorum sensing (QS), are virulence determinants that impact the outcome of PA infections (3, 5–7). Interaction with the host immune system via soluble and cell surface receptors (e.g., toll-like receptors) controls signaling molecules (e.g., cytokines) and modulates the host response, which impacts disease severity both by influencing the rate of bacterial clearance and by causing collateral damage to host tissues (3, 5–9).

Given the growing problem of antimicrobial resistance in PA (9–11), improving therapy has been designated a priority by the Antimicrobial Availability Task Force of the Infectious Diseases Society of America (2). Because of its resistance attributes, PA is the most common antibiotic-resistant pathogen isolated from VAP (12), with a significant attributable mortality (13, 14), even with early and optimal therapy (15). Unfortunately, the multifaceted resistance mechanisms possessed by PA have made the development of new antipseudomonal antibiotics challenging (16). Thus, there is a need for novel approaches for controlling these infections in the future.

Recent technological advances in areas, such as genomics, proteomics, and microscopy, have led to rapid progress in our understanding of PA pathogenicity. Scientists are now pushing these discoveries through the translational pipeline in the hope of developing new therapeutic agents useful in the treatment of PA infections. Although a large number of PA virulence determinants are being actively targeted (Table 1), here we will focus on four determinants: type III secretion, QS, biofilm formation, and flagella. We will highlight recent advances in our understanding of basic mechanisms underlying each of these virulence determinants and cite examples of how each is being targeted for therapeutic intervention.

Type III Secretion

PA secretes a number of toxins into the extracellular environment, but one

*See also p. 1826.

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Table 1. Virulence determinants of PA that have been targeted for therapeutic intervention

Virulence Determinant	Type	References Demonstrating Role in Pathogenicity[a]	Examples of Therapeutic Interventions	References Demonstrating Potential Utility[a]	Furthest Progress in Translational Efforts
Type IV pili	Surface appendage	Tang et al (125) Chi et al (126)	Active immunization	Kao et al127 Ohama et al (128)	Preclinical
Flagella	Surface appendage	Feldman et al (129) Balloy et al (130)	Active and passive immunization	Doring et al (124) Doring et al (131)	Phase III trial
Lipopolysaccharide	Outer membrane component	Danner et al (132) Moskowitz et al (133) Pier et al (134)	Active and passive immunization	Zuercher et al (135) Lang et al (136) Lai et al (137)	Phase III trial
Alginate	Cell surface exopolysaccharide	Simpson et al (138) Cabral et al (139)	Active and passive immunization	Kashef et al (140) Theilacker et al (141) Pier et al (142)	Phase I trial
Type III secretion	Secretion system	Shaver et al (20) Lee et al (143) Vance et al (144)	Active and passive immunization, small molecule inhibitors	Sawa et al (56) Neely et al (57)	Phase I/II trials
Elastase	Protease	Park et al (145) Azghani et al (146)	Active immunization	Matsumoto et al (147) Sokol et al (148)	Preclinical
Alkaline protease	Protease	Nicas et al (149) Guzzo et al (150)	Active immunization	Matsumoto et al (147)	Preclinical
Exotoxin A	Toxin	Nicas et al (149) Miyazaki et al (151)	Active and passive immunization	Denis-Mize et al (152) Hertle et al (153) El-Zaim et al (154)	Preclinical
Quorum-sensing	Cell-to-cell communication	Pearson et al (155) Rumbaugh et al (156)	Natural and synthetic inhibitors	See Table 2	Preclinical
Biofilms	Bacterial aggregates	Jesaitis et al (157) Cochran et al (158)	Antimicrobial coatings, small molecule inhibitors	See Table 3	Phase III trial

PA, *Pseudomonas aeruginosa*.
[a]References are not comprehensive but show representative studies from the field. Adapted from Refs. 159–162.

set of toxins is injected directly into host cells. This occurs through a macromolecular syringe called a type III secretion system (TTSS) (17). This system is important in pathogenesis in a number of animal models of infections (18–20). The TTSS of PA consists of 36 coordinately regulated genes that encode components of the secretion apparatus and a translocon, and factors that regulate secretion (17, 21). The secretion apparatus exports toxins from across the bacterial cell envelope, whereas the translocon is responsible for injecting these toxins into the host cell (Fig. 1). Three proteins, PcrV, PopB, and PopD, are necessary for assembly of a competent translocon (22, 23). The secreted toxins themselves are referred to as effector proteins, and four of them have been identified to date: ExoS, ExoT, ExoU, and ExoY. The first three have been closely linked to virulence and will be discussed here.

ExoS and ExoT are closely related bifunctional toxins that encode both Rho GTPase activating protein activity and ADP-ribosyltransferase activity (24–26). These activities work in concert to disrupt the host cell actin cytoskeleton, block phagocytosis, and cause cell death (27). Whereas the *exoT* gene is found in all PA strains, the *exoS* gene is present in approximately 70% of clinical isolates (28). Recent efforts have focused on the intracellular localization of ExoS. Once injected inside of host cells, ExoS localizes transiently to the plasma membrane and then traffics to the membranes of internal organelles, such as endosomes and the Golgi/endoplasmic reticulum (ER) (29). Intracellular membrane localization was critical for the ADP-ribosyltransferase activity, whereas plasma membrane localization was essential for the Rho GTPase activating protein activity of the toxin (29, 30). Interestingly, the ADP-ribosyltransferase activity portion of ExoS (and presumably also of ExoT) only becomes activated upon interaction with a host-derived cofactor identified as a 14-3-3 protein (also termed FAS for *F*actor *A*ctivating Exo*S*) (Fig. 1) (31–35). 14-3-3 proteins are abundant and serve as scaffold for the colocalization of numerous host cell constituents (36). ExoS, thus, illustrates the propensity of type III effector proteins to hijack host processes and factors, using them to subvert the injected cell.

ExoU is the most virulent of the PA type III effector proteins. The gene encoding this toxin is found in approximately 30% of clinical isolates (28). This potent cytotoxin encodes phospholipase A_2 activity but only after interaction with a host cell cofactor (37, 38). Recently Sato et al (39) demonstrated that superoxide dismutase 1 was such a cofactor (Fig. 1). Like ExoS, ExoU localizes to the plasma membrane but uses an unrelated membrane localization domain to do so (40). ExoU may damage host tissues in multiple ways. Its phospholipase A_2 activity leads to rapid cell death, perhaps by direct dissolution of the plasma membrane (37, 38, 41), but its phospholipase activity may also stoke the inflammatory fire during infection by generating arachidonic acid, which serve as substrate for the cyclooxygenase and lipoxygenase pathways (42). The net result is production of large amounts of prostaglandins, such as PGE_2 and PGI_2 (42), which may in turn contribute to the excessive inflammation, increased tissue damage, and bacterial dissemination of infections caused by ExoU-secreting strains (6).

Considerable progress has been made in understanding the regulation of type III secretion in PA. Previous research identified ExsA as a global activator of this system that binds to the promoters responsible for expression of type III se-

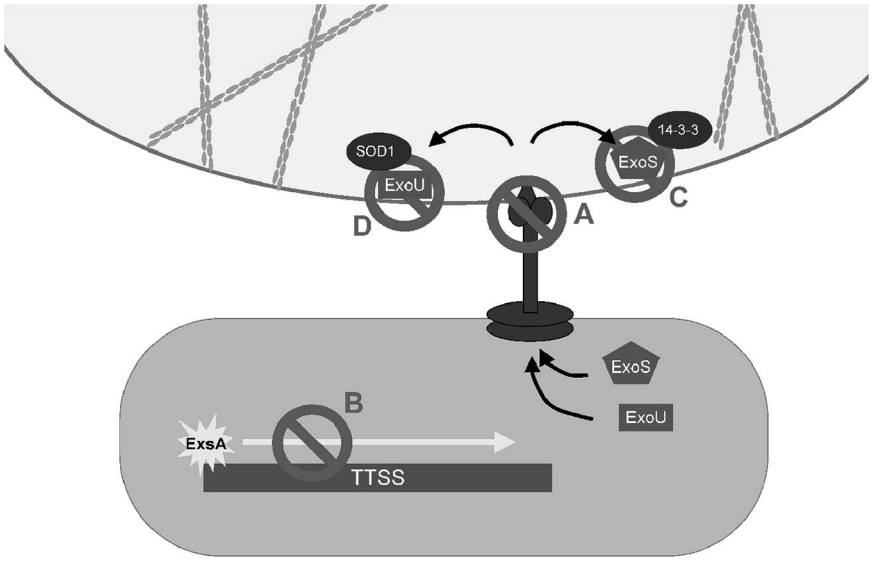

Figure 1. The type III secretion system of *Pseudomonas aeruginosa*. The transcriptional activator ExsA controls expression of the type III secretion regulon, including the genes encoding the effector proteins ExoS and ExoU. On contact with a host cell, induction of the type III secretion system (*TTSS*) occurs and secretion is activated. ExoS and ExoU are injected through the needle apparatus into the host cell, where they initially localize to the plasma membrane. At some point, each interacts with its respective cofactor (14-3-3 protein for ExoS, superoxide dismutase 1 [*SOD1*] for ExoU) and subsequently targets host cell substrates. Four points for potential therapeutic interventions are indicated: "A" represents the targeting of PcrV, a protein necessary for the translocation of effector proteins across the host cell plasma membrane, by antibodies. "B" represents the inhibition of ExsA binding to TTSS promoters by inhibitors. "C" and "D" represent inhibition by small molecules of the enzymatic activities of ExoS and ExoU, respectively.

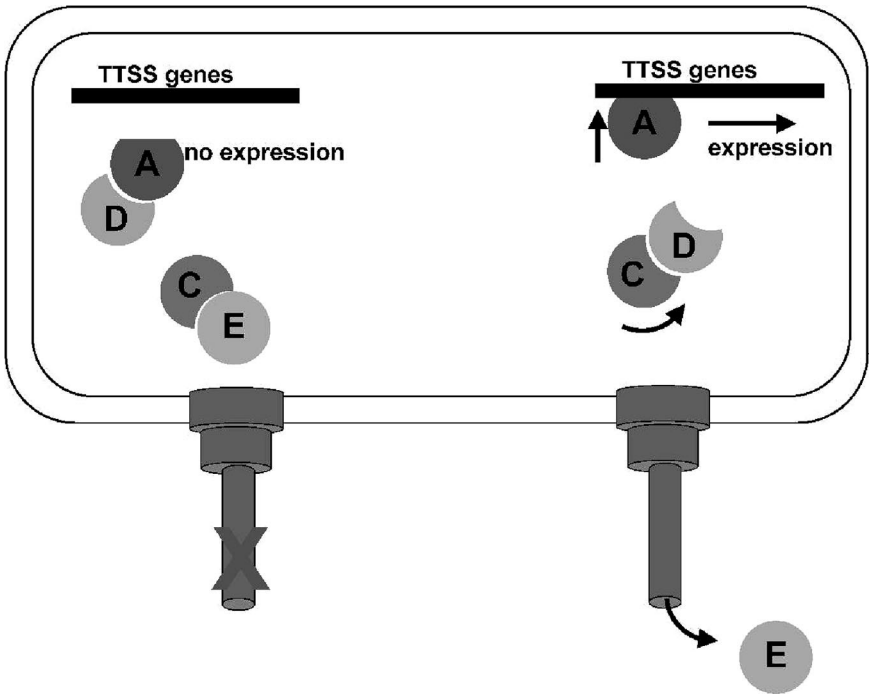

Figure 2. Regulation of *Pseudomonas aeruginosa* type III secretion genes. The left side of the figure represents regulation of type III secretion system (*TTSS*) genes in the absence of active protein secretion. In this case, ExsE ("E") is not secreted but rather is available to bind and sequester ExsC ("C"). This frees ExsD ("D") to bind and sequester ExsA ("A"), preventing ExsA from activating TTSS promoters and expressing TTSS genes. The right side of the figure represents regulation of TTSS genes during active secretion. In this situation, ExsE is secreted outside the bacterium through the type III secretion needle, freeing ExsC to bind and sequester ExsD. ExsD is, thus, unable to bind ExsA, which in turn is available to bind to TTSS promoters and facilitate expression of the corresponding genes. In this way, expression of TTSS genes is synchronized with secretion of TTSS proteins.

cretion genes (43, 44). The complex upstream regulatory network that controls ExsA is now being elucidated and involves at least three "catch and release" proteins (ExsC, ExsD, and ExsE) that work in concert to ensure that ExsA is only available to induce expression of this system when secretion is actively occurring (Fig. 2) (45–51).

Translation of our knowledge of the PA TTSS to the clinical setting is crucial for evaluating the potential of type III secretion neutralizing strategies as effective therapies. In this regard, it is promising that type III secretion proteins are expressed during human infections and that the results of studies of humans with PA infections mimic those of animal models (10–11, 52, 53). For example, the presence of a functional TTSS was associated with bacterial persistence in the lungs (and, therefore, perhaps clinical recurrence), higher relapse rates, and increased mortality in patients with acute respiratory infections caused by PA (10–11, 54). Furthermore, secretion of type III proteins was associated with increased mortality in patients with a high bacterial burden in respiratory secretions but who failed to meet clinical criteria for the diagnosis of VAP (55). Thus, the PA TTSS appears to have both pathogenic and prognostic significance in human infections.

Given the importance of type III secretion in the pathogenesis of PA infections, it is not surprising that efforts have been made to design reagents to disrupt it. A significant amount of effort has gone toward developing antibodies and vaccines that target the type III secretion apparatus itself and, therefore, prevent secretion (Fig. 1) (18, 56–61). Sawa et al (56) demonstrated that antibodies targeting PcrV, a protein believed to be located at the tip of the secretion apparatus, prevented effective type III secretion and resulted in increased survival and decreased lung injury in a mouse model of acute pneumonia. This same group found that passive anti-PcrV immunization protected against fatal PA challenge in a burned mouse model of infection (57). Using anti-PcrV IgG in a model of septic shock associated with *Pseudomonas*-induced lung injury also demonstrated a decrease in lung injury, bacteremia, and plasma tumor necrosis factor-α levels as well as improvement in hemodynamic parameters (58). Further clinical studies are underway to evaluate the usefulness of

Table 2. Inhibitors of PA quorum sensing

Class	Examples	Mechanism	References
Autoinducer analogs	Cyclopentanol, cyclopentylamide, and cyclohexanone compounds, tetrazole derivatives	Block autoinducer receptor	163–169, 100
Structurally unrelated autoinducer antagonists	4-Nitro-pyridine-*N*-oxide, triphenyl compound	Block autoinducer receptor	168, 169
Natural compounds	Products from fungi (penicillic acid), marine macroalga (furanone derivatives), garlic, medicinal plants	Decrease concentration of autoinducer receptor, unknown	84–87, 97, 98 168, 170–173
Enzymes	AHL-lactonase, AHL-acylase	Degrade autoinducers	82, 83
Antibiotics, metabolic compounds	Azithromycin, triclosan, *S*-adenosylhomocysteine, *S*-adenosylcysteine, sinefungin	Inhibit synthesis of autoinducer	81, 174, 175

PA, *Pseudomonas aeruginosa*; AHL, acyl homoserine lactone.

PcrV-specific antibodies as an adjunctive therapy in the clinical setting.

Efforts have also been directed at developing inhibitors that block other aspects of the PA TTSS. For example, small molecule inhibitors of ExoU's phospholipase A_2 activity and ExoS's ADP-ribosyltransferase activity activity have been identified and designated pseudolipasin and exosin, respectively (62, 63). Likewise, inhibitors of ExsA, a regulator necessary to express all TTSS genes, have also been identified (64). Each of these compounds provided protection from killing in models of PA infection.

Quorum Sensing

Like people, PA bacteria behave differently depending on whether they are alone or in a crowd. They accomplish this by using an intercellular signaling process called QS (65). In QS, small compounds called autoinducers are released by bacteria into the environment. Autoinducer concentrations are then sensed by neighboring bacteria to infer the density of the local bacterial population and to regulate gene expression accordingly. PA QS systems regulate about 350 genes (6% of the PA genome) and play a role in the regulation of a wide variety of processes including biofilm formation and production of numerous toxins (66–73). Given this regulatory breadth, it is not surprising that QS plays an essential role in virulence (74). Two primary QS systems were initially identified in PA, the *las* and the *rhl* systems (66–68, 75). More recently, a third QS system was identified in PA, referred to as the *Pseudomonas* Quinolone Signal (PQS) (76). PQS is controlled by *las* system and itself regulates the *rhl* system, suggesting that it acts as link between the two systems.

Just as many environmental organisms synthesize antibiotics to gain an advantage over microbial competitors, some also produce enzymes that degrade the QS autoinducer signals of other species of bacteria (77). Recent evidence suggests that mammalian cells too have developed such capabilities. Paraoxonases (PONs) are mammalian enzymes that are capable of degrading PA autoinducer molecules and thereby have the potential to disrupt QS (78–80). Treatment of PA with PON-containing serum inhibited biofilm formation, which requires functional QS (79). Thus, these enzymes may play an important role in host defense against PA.

Numerous approaches have been successfully used to inhibit QS in culture and *in vivo* model systems (Table 2). For example, triclosan, an antimicrobial substance used in soaps, toothpaste, cleansers, and deodorants, has been shown to inhibit the synthesis of autoinducer (81). The anti-QS strategies of bacteria themselves have been exploited. Expression of bacterial enzymes that degrade autoinducers resulted in decreased production of QS-regulated toxins by PA (82, 83). In another approach, natural and synthetic compounds have been screened for their utility in preventing the interaction between the autoinducer and its receptor. Much effort has been directed toward furanones, compounds produced by marine macroalga with anti-fouling properties (84). Although naturally occurring furanones lacked substantial activity, modified furanone compounds inhibited QS and increased bacterial clearance in a mouse model of infection (85–87). Further investigations are necessary to determine whether these approaches will prove efficacious in inhibiting QS in human infections.

Biofilms

Biofilms are bacterial cities, highly organized, microbial communities encased in a polysaccharide matrix and attached to a surface (88). When that surface is a surgical implant, endotracheal tube, catheter, or the airways of individuals with CF, biofilms become a medical problem. They are highly resistant to antimicrobial agents, which occurs by a number of mechanisms that are now becoming clear. When dispersed and grown on agar, a subpopulation of PA bacteria from biofilms will form dwarf colonies referred to as small-colony variants (SCVs). These colonies consist of highly adherent antibiotic-resistant variants implicated in persistent infections (89). Several groups have isolated SCVs of PA from biofilms as well as the respiratory tracts of individuals with CF (90–92). A genetic basis for antibiotic resistance of biofilms was identified by Mah et al (93). By performing a genetic screen for loss of antibiotic resistance in biofilms, they identified the gene *ndvB*. Biofilms formed by a PA strain containing a disrupted copy of the *ndvB* gene were more susceptible to antibiotics. The authors hypothesized that NdvB encodes a periplasmic glucan that physically interacts with and sequesters antibiotics, preventing them from reaching their target sites (93).

Given their importance in PA pathogenesis, biofilms have been an obvious target for efforts aimed at therapeutic interventions (Table 3). One approach has been to block the earliest step in biofilm

Table 3. Approaches to preventing or disrupting PA biofilms

Approach	Examples	Mechanism	References
Antimicrobial coating of medical devices	Silver, chlorhexidine	Prevent biofilm formation by killing bacteria and preventing bacterial adherence	94, 95, 176
Iron limitation	Lactoferrin, transferrin	Prevent adherent bacteria from forming biofilms	96, 177
Iron excess	$FeCl_3$, $Fe_2(SO4)_3$	Prevent biofilm formation, disrupt preformed biofilms	178, 179
QS inhibitors	Furanone, patulin and penicillic, plant lactones	Prevent biofilm formation, decrease biofilm resistance to tobramycin	97, 98, 100, 85, 86, 180
Subinhibitory levels of antibiotics	Macrolides, mupirocin	Delay biofilm formation, decrease biofilm resistance to tobramycin, alter biofilm architecture, decrease polysaccharide content	181–184
Inhibitors from natural products	Marine alkaloid derivatives, ursene triterpenes from tropical tree	Prevent biofilm formation, disperse preformed biofilms	185–187
Inhibitors from random compound screens		Prevent biofilm attachment and formation	188
Metal chelators	EDTA	Killing and dispersal of bacteria in biofilms	101
Electrical current		Enhanced susceptibility of biofilm to biocides	104, 105
Mucolytic agent	Ambroxol	Decreased synthesis of alginate	189
Degradation of polysaccharide	Alginate lyase	Enhanced susceptibility of biofilm to aminoglycosides	102, 103
Activation of endogenous dispersal mechanisms	Nitric oxide	Disruption of preformed biofilms	106

PA, *Pseudomonas aeruginosa*.

formation: bacterial attachment. For example, coating of endotracheal tubes with silver was associated with a delay in bacterial colonization, reduced bacterial burdens in mechanically ventilated patients, and a reduction in the incidence of VAP (94, 95). Several groups have targeted the processes necessary for biofilms to evolve into mature structures, such as quorum-sensing and iron acquisition (96, 97). Others have identified natural or synthetic compounds that prevent biofilm formation (98–100). Efforts have also been directed at disrupting already formed biofilms by using compounds toxic to bacteria within these structures (101), by degrading the polysaccharide matrix (102, 103), by applying electrical current (104, 105), and by inducing bacterial dispersal from biofilms (106). Each of these approaches has the potential to be prophylactically or therapeutically useful.

Flagella

The flagellum of PA is required for swimming motility but also plays crucial roles in biofilm dispersal and adhesion to the surface of host cells (107–109). The significance of these processes in pathogenesis is underscored by the loss of virulence of nonflagellated mutants in models of acute infection (110, 111). During infection flagellin, the primary structural component of the flagellum is recognized by Toll-like receptor 5 on the surface of host cells. Thus, toll-like receptor 5 is used by the host as a surveillance mechanism to detect invading PA bacteria and in turn trigger the immune response by inducing the synthesis of cytokines, such as tumor necrosis factor, interleukin-6, and interleukin-8 (112–115).

Despite the importance of flagella in acute infection, PA actually down-regulates expression of flagellin over the course of chronic infection in the CF lung, perhaps to evade the host immune response (116, 117). The mechanism of this down-regulation is now being elucidated and is reminiscent of that described for type III secretion (Fig. 2). Elastase released by neutrophils in respiratory mucus degrades the flagellar hook protein FlgE at the bacterial surface (118). In the absence of FlgE, the flagellar apparatus is no longer competent for export, and the otherwise secreted anti-sigma factor FliM accumulates within the bacterium and binds to FliA. Sequestration of FliA prevents expression of flagellar genes normally targeted by this transcriptional activator, resulting in the absence of flagella (119). An additional mechanism by which flagella are down-regulated during chronic infection is the accumulation of mutations in the *fleQ* gene, which encodes a major regulator of the flagellar regulon (120).

Although 40% of PA isolates from patients with CF do not produce flagella, this virulence factor is still thought to be necessary for the initial infection of these patients (121). Animal models have demonstrated that antibodies against the flagellum, induced by either active or passive immunization, are protective (122, 123). Prevention of PA lung infection by immunization against flagellar antigens might, therefore, be a suitable adjunctive therapy in individuals with CF. A recent randomized placebo-controlled trial of 483 patients with CF found a 34% reduction in infection episodes over a 2-year period in those immunized with a bivalent flagella vaccine (124).

Future Directions

It is anticipated that novel therapeutic interventions based on PA virulence factors (Table 1) will become a part of standard clinical practice. These interventions will take one of two forms: First is vaccination of high-risk patient populations. Further studies will be necessary to evaluate the role of flagella vaccines in specific patient groups and to further define the dose and immunization schedule for optimal induction of long-lasting serum titers of antiflagellar IgG. Second, immunotherapies and inhibitors may be useful agents in the prevention of high burden colonization or the treatment of

infection. For example, such agents may be administered alone to colonized patients undergoing mechanical ventilation or as adjuncts to conventional antibiotics in patients who have already developed VAP. Although much additional work is required, the utility of targeting PA virulence factors is already being borne out by such interventions as silver-coated endotracheal tubes and the flagella vaccine.

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