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MINIREVIEW

Pouring Salt on a Wound: *Pseudomonas aeruginosa* Virulence Factors Alter Na^+ and Cl^- Flux in the Lung

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***Pseudomonas aeruginosa* is a ubiquitous opportunistic pathogen with multiple niches in the human body, including the lung. *P. aeruginosa* infections are particularly damaging or fatal for patients with ventilator-associated pneumonia, chronic obstructive pulmonary disease, and cystic fibrosis (CF). To establish an infection, *P. aeruginosa* relies on a suite of virulence factors, including lipopolysaccharide, phospholipases, exoproteases, phenazines, outer membrane vesicles, type III secreted effectors, flagella, and pili. These factors not only damage the epithelial cell lining but also induce changes in cell physiology and function such as cell shape, membrane permeability, and protein synthesis. While such virulence factors are important in initial infection, many become dysregulated or nonfunctional during the course of chronic infection. Recent work on the virulence factors alkaline protease (AprA) and CF transmembrane conductance regulator inhibitory factor (Cif) show that *P. aeruginosa* also perturbs epithelial ion transport and osmosis, which may be important for the long-term survival of this microbe in the lung. Here we discuss the literature regarding host physiology-altering virulence factors with a focus on Cif and AprA and their potential roles in chronic infection and immune evasion.**

Pseudomonas aeruginosa is a Gram-negative gammaproteobacterium that is present in diverse environments and is a common opportunistic pathogen displaying high-level antibiotic resistance and with the capability of infecting many hosts, including humans. In humans, these infections tend to occur in association with epithelial cell damage to the skin or eye or medical devices such as catheters or ventilators or in immunocompromised individuals. In addition to these illnesses, *P. aeruginosa* lung infections are common in individuals with chronic obstructive pulmonary disease (COPD), ventilator-associated pneumonia (VAP), and cystic fibrosis (CF) (1).

COPD is caused primarily by tobacco smoke inhalation. Long-term use of tobacco products leads to an increase in airway inflammation and a breach of the airway/vascular barrier (2), which in turn leads to chronic bronchitis, airway remodeling, and emphysema, resulting in decreased oxygenation of the blood and a reduced forced expiratory volume in 1 s, the hallmark of COPD. Patients with this inflammatory disease are at greater risk of microbial infection. For patients with COPD, *P. aeruginosa* can cause a short-term infection that is cleared quickly, induce severe exacerbations, or chronically colonize the lung (reviewed in references 3 and 4).

Nosocomial infections such as VAP, caused by intubation of an individual, are a growing problem, with mortality rates as high as 13 to 55% (5, 6). Mechanical ventilation is thought to readily permit the passage of bacteria, which may be attached to the ventilator tube, to the lower airways, and because VAP patients are often sedated or immobile, the diagnosis of an infection can be delayed. The bacteria that most commonly cause VAP include members of the family *Enterobacteraceae*, *Staphylococcus aureus*, and *P. aeruginosa*. *P. aeruginosa* infections are of particular concern, as they are associated with a mortality rate as high as 70 to 80% (7).

In the case of CF, patients have a mutation in the gene encoding the CF transmembrane conductance regulator (CFTR). CFTR is a chloride ion channel of the ABC transporter family, and mutations in CFTR result in misfolding, a lack of proper localization, and/or a complete lack of the protein. CFTR, in cooperation with the epithelial sodium channel (ENaC), is responsible for controlling the level of airway surface liquid (ASL) (Fig. 1). ASL is the periciliary liquid layer, which is critical for the removal of inhaled contaminants such as bacteria in that it provides hydration to lung mucus and a substrate for ciliary movement (8) (Fig. 1).

In addition to its role in transporting Cl^- ions, CFTR activity is known to reduce ENaC activity, and thus, the absence of CFTR leads to ENaC hyperactivity (9). The CFTR-mediated regulation of ENaC appears to occur regardless of the chloride concentration within the cell (10), although the mechanism of repression is controversial (reviewed in references 11 to 13). Interaction of these two proteins, either directly or indirectly as part of a larger protein complex, is the currently favored model, as yeast two-hybrid, immunoprecipitation, and fluorescence resonance energy transfer analyses support such interactions (14–16).

Thus, depletion of CFTR results in a loss of Cl^- secretion and an increase in sodium import (due to an increase in ENaC activity). The combined effects of CFTR loss and ENaC derepression are a reduction of ASL height and an associated thickening of mucus and ciliostasis (8), although the precise mechanisms by which these changes occur is still somewhat controversial (11). The altered airway environment in CF becomes a setting in which *P. aeruginosa* can eventually establish an infection.

ESTABLISHING AN INFECTION

The lung is a hostile environment in which to initiate an infection; thus, *P. aeruginosa* possesses a cache of virulence factors to manipulate host physiology and overcome host defenses. These virulence determinants are both secreted and cell associated. Flagella, pili, and lipopolysaccharide are not only important for motility and adhesion but also serve as activators of Toll-like receptor 5

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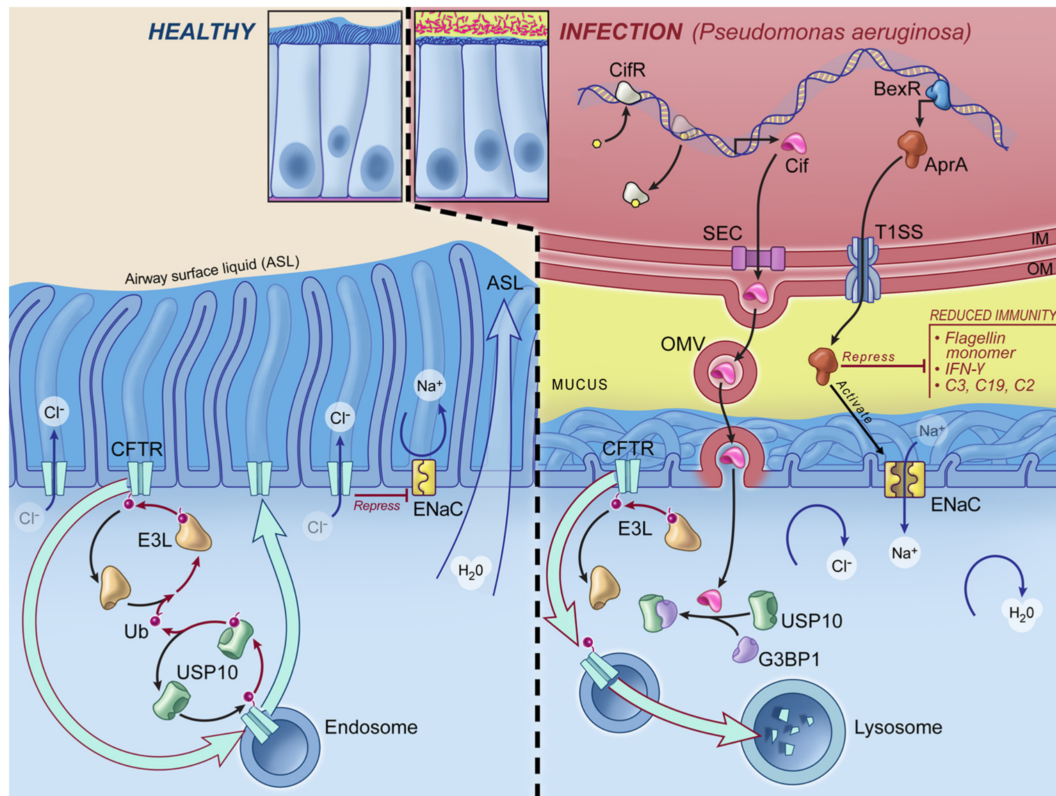

FIG 1 Effects of AprA and Cif on host cell physiology. In the absence of *P. aeruginosa* (left), CFTR is recycled at the apical membrane through ubiquitination by an E3 ligase (E3L) and deubiquitinated by USP10. CFTR performs two functions, chloride secretion and repression of ENaC, a sodium importer. Normal CFTR function promotes an osmotic gradient that facilitates hydration of the ASL, providing a liquid for ciliary movement. When *P. aeruginosa* is present (right), Cif is expressed, likely in response to endogenous epoxides (yellow circles), and interacts with the repressor protein CifR to derepress *cif* gene transcription. Cif protein is secreted via the Sec secretion system (SEC) and can be delivered directly to the host cell or via OMVs, which have been shown to fuse with lipid rafts to release their contents into the cytoplasm. Cif stabilizes an interaction between G3BP1 and USP10, which in turn prevents USP10 from deubiquitinating CFTR, resulting in the shunting of CFTR to the lysosome for degradation. Reduced CFTR also eliminates a key mechanism of ENaC repression. The LysR-type regulator BexR positively regulates the transcription of the *aprA* gene. The AprA protein is secreted via the T1SS that is encoded by three genes found adjacent to the *aprA* gene. AprA has been shown to proteolytically degrade the flagellin monomer, a potent TLR5 activator, as well as IFN-γ and complement proteins, all of which are important for activation of the immune response. Additionally, AprA can proteolytically activate ENaC, which increases sodium import into the host cell. Thus, in the presence of *P. aeruginosa*, CFTR is degraded and ENaC activity in increased, which dramatically shifts the osmotic flux toward the cell, resulting in dehydration of the ASL and ciliostasis. Ub, ubiquitin; IM, inner membrane. Illustration ©2013 William Scavone, Kestrel Studio, reprinted with permission.

(TLR5), TLR2, and TLR4, which in turn lead to immune activation (17, 18). Additionally, LepA, a protease, cleaves protease-activated receptors 1, 2, and 4 to activate NF-κB and increase inflammation (19). Rhamnolipids consist of a mixture of secreted surfactants that promote ciliostasis (20). Phenazines, exported redox-active molecules, are thought to be important for *Pseudomonas* defense against the host and as a terminal electron acceptor for respiratory growth. Furthermore, these molecules negatively impact a number of eukaryotic cellular processes, including respiration, electron transport, and gene expression (reviewed in reference 21). Indeed, phenazines are correlated with a poorer prognosis in CF (22). *P. aeruginosa* also has the ability to halt epithelial cell protein expression and kill host cells by using the ADP-ribosylating protein ExoA (reviewed in references 23 to 25). The type III secretion system (T3SS) effectors have also been well studied and recognized as key for establishing infection (reviewed in references 26 and 27). These T3SS effectors include ExoS and ExoT, both of which have ADP-ribosyltransferase and GTPase-activating activities. ExoS and ExoT work in concert to inhibit actin polymerization, prevent phagocytosis and cell migration, and promote apoptosis (28). Similarly, the T3SS-delivered effector ExoY impairs actin polymerization but also increases membrane permeability (29), while ExoU is a phospholipase that can cause membrane damage and cell lysis and modulate the inflammatory response (24, 30). Together, these proteins dramatically alter the epithelial layer of the lung, disrupting cell polarity, inducing damage, and preventing *P. aeruginosa* endocytosis and clearance (31), thereby allowing this microbe to establish an infection in the lungs.

INFECTION MAINTENANCE

Once *P. aeruginosa* has invaded the lung and inhibited clearance, it must induce changes to reduce immune activation and obstruct clearance mechanisms to persist in the lung. To facilitate mucus penetration, *P. aeruginosa* employs a suite of secreted enzymes (exoproteins) to dampen host immunity (reviewed in references 26 and 32). These immunosuppressing factors include the elastases LasA and LasB. LasA is responsible for inducing syndecan (co-receptor proteins) shedding from cells, which has been shown to be important for *P. aeruginosa* lung survival (33). LasB cleaves the

abundant elastin in the lung that is required for normal lung elasticity, as well as surfactant protein D, a collectin that is an important modulator of immune effector cell function (34). Protease IV also degrades surfactant proteins A, B, and D, which are important for surface tension and innate immunity (35). The phospholipases PlcB, PlcH, and PlcN target the mucus layer and cell membrane, facilitating bacterial transit through the mucus layer and liberating nutrients exploited by the bacteria (36–38). Furthermore, PlcH has been shown to suppress the neutrophil respiratory burst, which may also facilitate *P. aeruginosa* survival (39). A small, uncharacterized secreted factor (>3 kDa) produced by *P. aeruginosa* has also been shown to suppress interleukin-8 and NF-κB expression from epithelial cells (40), thus damping the typical inflammatory response to pathogens. Along with these extracellular proteins, production of the polysaccharide alginate increases in many CF strains, which in turn stimulates mucin production, thereby limiting immune recognition and clearance (41–43).

The expression of all of the virulence factors listed above is critical for *P. aeruginosa* to establish and maintain an infection and avoid clearance early in the infection process; however, many of these virulence factors are lost during chronic infection, in part to evade recognition and reduce inflammasome activation (4, 44–46). This loss reflects the adaptation of *P. aeruginosa* to the lung and a transition to a chronic lifestyle.

CIF, A NOVEL VIRULENCE FACTOR

Interestingly, a novel virulence factor of *P. aeruginosa* that was identified fairly recently does not appear to be absent from isolates harvested from the CF lung over time, unlike many of the other virulence factors discussed in the previous section (47). This virulence factor was first identified as a secreted ~36-kDa protein that reduces chloride secretion by epithelial cells and was subsequently named CFTR inhibitory factor (Cif) (48).

Early studies suggested that Cif was an epoxide hydrolase (49). This activity was later confirmed by further enzymatic analysis, as well as crystallographic studies (50, 51). In fact, Cif has an unusual active site and is the first described epoxide hydrolase of its class (Fig. 2) (52). We believe this epoxide hydrolase activity is important for the Cif-mediated effect on CFTR, as a mutation just outside the active-site tunnel eliminates the epithelial cell activity of Cif (41, 44). Ongoing studies are aimed at verifying this hypothesis.

Cif was initially shown to reduce apical membrane CFTR, and the mechanism by which this altered CFTR expression occurs was recently elucidated. Typically, CFTR is efficiently recycled by endocytosis at the apical face of the epithelium (53), which is due to ubiquitination of CFTR by a yet-to-be defined E3 ligase. This process of recycling ensures that proper protein folding is maintained (54). CFTR is then deubiquitinated by USP10, and the endosome containing CFTR is conveyed to the apical membrane, where CFTR can once again play its role as an ion transporter and regulator of ENaC. If Cif enters the host cell, it stabilizes the interaction of USP10 and its negative regulatory protein G3BP1, preventing USP10 activity (55). The precise mechanism by which Cif mediates the USP10-G3BP1 interaction is unknown. CFTR is not deubiquitinated in Cif-exposed epithelium, and the fate of CFTR is to be shunted to the lysosome for degradation (Fig. 1). Cif has also been shown to reduce the epithelial cell expression of another ABC transporter, P-glycoprotein, a drug efflux pump highly expressed in cancer; however, other drug efflux ABC transporters like MPR1 and MPR2 are not affected by Cif (56). These studies have been performed with cultured epithelial cells, which may limit the possible range of Cif targets; thus, additional Cif virulence effects may be seen in an animal model. Intriguingly, microarray studies indicate that Cif is highly expressed in a rat peritoneal infection model, which may indicate a role in systemic infection (57).

Cif expression is regulated at the transcriptional level by a TetR family repressor called CifR (58). The CifR protein binds to two regions in the intergenic space between the *cifR* gene and the operon containing the *cif* gene, overlapping their respective promoters and transcriptional start sites, to bidirectionally repress transcription (47). Epoxides act as both substrates for and inducers of Cif, as CifR repression is alleviated in the presence of epoxides (58), although there appears to be some specificity of inducers (47). Interestingly, the *cifR* transcript appears to be highly expressed in CF sputum, suggesting that *P. aeruginosa* encounters epoxides in the lung (47).

P. aeruginosa is not thought to interact directly with the epithelial cell layer during chronic infection but, instead, likely resides within the mucus layer above the ASL (59). The distance between the bacteria and the epithelial cell makes transmission of bacterial proteins a challenge. Cif is packaged into outer membrane vesicles (OMVs), as well as directly secreted (49, 51). These OMVs have been shown to diffuse through the mucus layer and fuse with lipid rafts within the membrane to deliver Cif to the host cytoplasm (60). OMV-mediated Cif delivery is very effective because 17,000-fold less protein is required for equivalent CFTR reduction than when the purified protein is applied directly (60). Therefore, *P. aeruginosa* has developed an effective means of de-

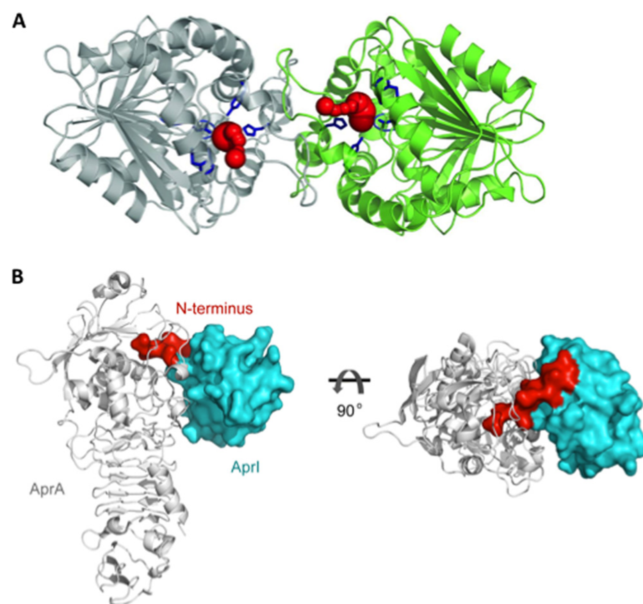

FIG 2 Structures of Cif and AprA. (A) Cif represents a novel class of α/β hydrolase. Shown is the ribbon structure of a Cif homodimer with the side chains of active residues depicted in blue. The tunnel to the active site is shown in red. (Adapted from reference 50.). (B) AprA-AprI interaction. Crystal structure of AprA (gray, ribbon) interacting with its inhibitor, AprI (blue, surface representation). The red region of AprI indicates the N-terminal portion that interacts with the active-site cleft of AprA. (Adapted from reference 78 with permission of the publisher.)

livering this toxin and reducing chloride secretion across the epithelium.

Reduction of CFTR has other physiological implications in addition to inhibition of chloride secretion and derepression of ENaC. Reduction or loss of CFTR inhibits the microbicidal activity of neutrophils by limiting chloride import to endosomes and preventing hypochlorous acid formation in *P. aeruginosa*-containing vesicles (61, 62). Thus, Cif-mediated reduction of CFTR could also serve as a means of innate immune evasion by *P. aeruginosa*.

APRA, A MULTIFUNCTIONAL PROTEASE

Another pseudomonal protein shown to be important for phagocytic evasion is alkaline protease (63). Alkaline protease (AprA, Fig. 2B) is a zinc metalloprotease produced by *P. aeruginosa* that has long been understood to be important for virulence (64–66). This secreted serralysin family protease was first isolated from culture supernatants in 1963, and its expression appears to be maintained in many clinical isolates (44, 67). High levels of AprA expression have been correlated with *P. aeruginosa* infections of the eye, the gastrointestinal tract, and wounds (68). High expression has also been correlated with mucoidy and implicated in pulmonary exacerbation in CF (69, 70).

The structure of the ~50-kDa AprA protein was elucidated in 1993 and revealed a protein with two domains (71). The N-terminal domain is the proteolytic region that coordinates a Zn^{2+} in its active-site cleft, while the C-terminal domain contains a number of repeats (RTX motif) that bind eight Ca^{2+} ions, as well as the secretion signal (71). The C-terminal domain's interaction with Ca^{2+} is thought to be important in proper protein folding after secretion. Indeed, an increase in extracellular AprA has been observed in *P. aeruginosa* biofilms grown with high levels of calcium (72).

AprA is secreted by a complex of three proteins, AprD, AprE, and AprF, that form a T1SS. The genes encoding this T1SS are located next to the *aprA* gene on the chromosome (73). AprD is an ABC transporter predicted to be localized to the inner membrane, recognizes the signal sequence on AprA, and initiates transport across the membrane (26). AprE is the adaptor protein that transits the periplasm and connects AprD to AprF, the outer membrane pore protein (74). This secretion apparatus appears to be specific for AprA and AprX, a protein of unknown function (75). AprA has not been described as delivered via OMVs, although this is a possibility, as other described exoproteases have been shown to be present in vesicles (76).

Also located in the genomic vicinity of the *aprA* gene (just downstream) is the gene encoding AprI. AprI is an AprA inhibitor protein that is not secreted but instead remains in the periplasm, presumably to inhibit AprA-based proteolysis in this compartment as the protease is secreted. AprI has an N-terminal protrusion that has a high affinity for the AprA active site (K_d = 4 pM) and serves as a potent and specific inhibitor (Fig. 2B) (77, 78).

The *aprA* gene is activated by two transcriptional regulators. The quorum-sensing regulator, LasR, has been shown to increase the level of *aprA* transcript in an acylated homoserine lactone-dependent fashion (79, 80). However, it is not clear whether this regulation is direct or indirect. More recently, a LysR-type activator of the *apr* genes, BexR, was identified (81). This regulator binds directly to the *apr* genes to upregulate the transcription of the *apr* locus. BexR exhibits positive autoregulation, resulting in bistable expression of the loci regulated by this protein, including the *aprA* gene (81). Transcription of AprA is also activated by the sigma factor PvdS during iron starvation (82).

The somewhat closed structure of the AprA catalytic domain suggests that it has some degree of target specificity (78); however, the protease can degrade a number of bacterial and host proteins for immune recognition evasion (Fig. 1). AprA has been shown to aid in *P. aeruginosa* survival in the lung by cleaving transferrin to facilitate iron acquisition by siderophores (83), as well as inhibiting immune recognition by cleaving flagellin monomers to prevent TLR5 recognition (84). Furthermore, AprA degrades complement proteins C1q, C2, and C3, as well as gamma interferon (IFN-γ) (63, 85, 86), and loss of complement proteins has been shown to block phagocytosis and killing by neutrophils (63). Thus, it appears that AprA degrades many extracellular proteins that may limit the life span of *P. aeruginosa* in the lung.

Recently, Butterworth et al. also showed that AprA contributes to lung infection by proteolytically activating ENaC (87). This study showed that in the presence of AprA, Na^+ transport increased on both CF and non-CF cells, a finding with important implications for host cell sodium regulation. The authors concluded that this increase in Na^+ occurs at the membrane via cleavage of a sporadically exposed site, although direct evidence for this idea is currently lacking (87).

POTENTIAL IMPACTS OF CFTR AND ENAC MISREGULATION ON OSMOSIS

Loss of CFTR has been shown to broadly impact the biology of the lung epithelium, and the best documented of these effects is the loss of chloride secretion across the apical membrane. Cif is capable of shunting endocytosed CFTR to the lysosome, resulting in reduced CFTR. Given that CFTR has also been shown to be important for the proper regulation of ENaC, it is likely then that loss of CFTR due to Cif could result in increased ENaC activation. Furthermore, the secreted protease AprA has been shown to proteolytically cleave and activate ENaC above the level of untreated cells, exacerbating the perturbations of Na^+ and Cl^- homeostasis. Thus, *P. aeruginosa* employs a two-pronged approach to reduce ion transport to the ASL and dehydrate mucus (Fig. 1).

What are the consequences of altering the Na^+ and Cl^- balance via altering CFTR and ENAC levels and/or function? In CF, where the CFTR levels of many patients are already quite low, one might argue that further loss of CFTR might not have a clinically meaningful impact. However, recent recognition that even partial rescue of CFTR activity helps improve patient outcomes (88) indicates that reducing residual CFTR activity might do more harm than previously recognized. The action of Cif and AprA may also exacerbate the conditions of patients with less severe CFTR alleles. Additionally, AprA and Cif function could limit the effects of ENaC- and CFTR-targeted drugs for CF treatment. For example, it may be prudent to consider the effects of these *P. aeruginosa* virulence factors in drug design. CFTR potentiators and activators currently being developed may not have the desired degree of effect in the presence of Cif. Additionally, AprA may reduce the efficacy of ENaC inhibitors like benzamil.

Finally, and more broadly, loss of CFTR due to Cif and AprA-mediated activation of ENaC may be able to alter conditions sufficiently in the lung to transiently induce a CF-like state in patients with VAP or COPD, thus allowing colonization of the lung by this pathogen. Given that COPD is estimated to be among the most

prevalent diseases in the coming decades (2) and the high mortality rate of *P. aeruginosa*-associated VAP (5), our understanding of the complex microbe-host interactions in such diseases will be increasingly important. That is, development of Cif or AprA inhibitors for coadministration with antibiotics may help to improve the outcomes of patients with *P. aeruginosa* lung infections.

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