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Infection of human mucosal tissue by *Pseudomonas aeruginosa* requires sequential and mutually dependent virulence factors and a novel pilus-associated adhesin

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Summary

Tissue damage predisposes humans to life-threatening disseminating infection by the opportunistic pathogen *Pseudomonas aeruginosa*. Bacterial adherence to host tissue is a critical first step in this infection process. It is well established that *P. aeruginosa* attachment to host cells involves type IV pili (TFP), which are retractile surface fibers. The molecular details of attachment and the identity of the bacterial adhesin and host receptor remain controversial. Using a mucosal epithelium model system derived from primary human tissue, we show that the pilus-associated protein PilY1 is required for bacterial adherence. We establish that *P. aeruginosa* preferentially binds to exposed basolateral host cell surfaces, providing a mechanistic explanation for opportunistic infection of damaged tissue. Further, we demonstrate that invasion and fulminant infection of intact host tissue requires the coordinated and mutually dependent action of multiple bacterial factors, including pilus fiber retraction and the host cell intoxication system, termed type III secretion. Our findings offer new and important insights into the complex interactions between a pathogen and its human host and provide compelling evidence that PilY1 serves as the principal *P. aeruginosa* adhesin for human tissue and that it specifically recognizes a host receptor localized or enriched on basolateral epithelial cell surfaces.

Introduction

Pseudomonas aeruginosa, an environmental bacterium, is a major source of fatal nosocomial infections and the primary cause of morbidity and mortality in patients with cystic fibrosis (CF). Surgery, severe wounds, burns and corneal abrasion predispose otherwise healthy individuals to disseminating tissue infection (Garau *et al.*, 2003, Driscoll *et al.*, 2007). The ability of *P. aeruginosa* to cause infection requires multiple virulence factors including type IV pili (TFP) and a contact-dependent type III secretion system (T3SS) (Sadikot *et al.*, 2005). TFP are filamentous surface appendages expressed by a wide variety of human, animal and plant pathogens (Pelicic, 2008). In *P. aeruginosa*, TFP are

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specifically involved in adherence to host tissue, virulence and the formation of biofilms (Chi *et al.*, 1991, Tang *et al.*, 1995, O'Toole *et al.*, 1998). In addition, *P. aeruginosa* TFP mediate a form of surface-associated bacterial movement known as twitching motility through extension and retraction of pilus fibers (Skerker *et al.*, 2001).

The assembly and function of TFP in *P. aeruginosa* involves more than 40 gene products (Mattick, 2002). TFP are polymers composed primarily of a single repeating subunit termed pilin, which is encoded by the *pilA* gene (Strom *et al.*, 1986). Pilin contains an extended, hydrophobic N-terminal α-helical region followed by a globular C-terminal domain terminating in a disulfide-bonded loop (Hazes *et al.*, 2000, Craig *et al.*, 2003). Pilin subunits are predicted to assemble such that the hydrophobic N-terminal α-helical regions form the core of the pilus, and the globular C-terminal domains are exposed on the exterior surface of the fiber (Craig *et al.*, 2003). The pilus is assembled on the periplasmic surface of the inner membrane and extruded across the outer membrane via a pore complex composed of the secretin protein PilQ (Wolfgang *et al.*, 2000, Collins *et al.*, 2001). Although pilin is the major structural component of the pilus, other proteins are associated with the fiber and may play structural or functional roles. In *Neisseria gonorrhoeae*, six “pilin-like” proteins, sharing the highly conserved N-terminal α-helical region of pilin (PilH, PilI, PilJ, PilK, PilL, and PilV), are associated with the TFP fraction (Winther-Larsen *et al.*, 2005). PilX, a *N. meningitidis* homolog of *N. gonorrhoeae* PilL, is tightly associated with pilus fibers and is proposed to be a minor TFP subunit (Helaine *et al.*, 2007). An orthologous set of pilin-like proteins (FimU, PilV, PilW, PilX, PilE) is required for TFP biogenesis in *P. aeruginosa* (Alm *et al.*, 1997); however, their association with the pilus fiber and specific function remain to be determined.

Extension and retraction of TFP involves two cytoplasmic membrane-associated ATPases, PilB and PilT. PilB is required for the assembly of pilin monomers into mature fibers (Turner *et al.*, 1993, Chiang *et al.*, 2008), while PilT is involved in the disassembly of polymerized pilin and subsequent retraction of TFP (Whitchurch *et al.*, 1991, Chiang *et al.*, 2008). Loss-of-function mutations in *pilT* results in hyperpiliation and loss of twitching motility due to the inability of formed pilus fibers to retract (Whitchurch *et al.*, 1991). Inactivation of *pilT* was reported to result in the loss of cytotoxicity *in vitro*, presumably due to the inability of the TFP to retract and facilitate intimate contact between the bacteria and host cells, a process required for activation of the *P. aeruginosa* T3SS and subsequent delivery of anti-host effector proteins (Comolli *et al.*, 1999a, Vallis *et al.*, 1999, Sundin *et al.*, 2002). Mutants lacking *pilT* are capable of establishing pulmonary infection in mice, but are unable to spread to peripheral organs, presumably due to their inability to undergo twitching motility and disseminate (Comolli *et al.*, 1999a). Similarly, it has been shown that *pilT* mutants are unable to invade damaged corneal tissue *in vivo* (Zolfaghar *et al.*, 2003).

In addition to their function in twitching motility, TFP also mediate adherence of *P. aeruginosa* to eukaryotic cells. While the molecular basis for this interaction has been the subject of many studies, the actual mechanisms underlying TFP-mediated *P. aeruginosa* adhesion remain controversial. Previous studies suggest that *P. aeruginosa* pilin may be directly involved in bacterial adherence to host cells. Specifically, purified *P. aeruginosa* pili bound to the GalNAcβ1-4Gal moiety of the non-sialylated glycosphingolipids asialo-GM1 and asialo-GM2, which are abundant on the apical surface of mammalian epithelial cells (Lee *et al.*, 1994, Sheth *et al.*, 1994). Fab fragments generated from monoclonal antibodies specific for the exposed C-terminal disulfide-bonded loop (DSL) region of pilin and a synthetic peptide corresponding to the C-terminal DSL, were reported to inhibit adhesion of purified *P. aeruginosa* TFP to human buccal epithelial cells and the disaccharide GalNAcβ1-4Gal, respectively (Doig *et al.*, 1990, Sheth *et al.*, 1994, Schweizer *et al.*, 1998). Structural modeling suggested that the adhesive moiety of TFP (responsible for

asialylated glycosphingolipid binding) is likely only exposed at the distal tip of purified TFP fibers (Lee *et al.*, 1994). Based on these studies, it is widely assumed that the exposed C-terminal DSL of pilin serves as the primary *P. aeruginosa* adhesin for human tissue. However, more recent results have raised questions as to the role of pilin in TFP-mediated adherence as well as the identity of the pilus receptor. Specifically, Emam *et al*. (2006) showed that even though purified pili bound to asialo-GM1 or asialo-GM2, piliated *P. aeruginosa* failed to recognize the same receptor molecules. Second, it has been established that *P. aeruginosa* preferentially binds basolateral surfaces of epithelial cell monolayers or damaged areas of the monolayer where basolateral surfaces are exposed (Fleiszig *et al.*, 1997, Lee *et al.*, 1999). The fact that asialo-GM1 is predominantly localized to the apical compartment of polarized airway epithelial cells (Soong *et al.*, 2004) suggests that asialo-GM1 may not be the primary *P. aeruginosa* host cell receptor. Finally, we recently showed that adherence of *N. gonorrhoeae*, expressing TFP composed of *P. aeruginosa* pilin, to primary human epithelial cells was dependent on the gonococcal TFP-associated adhesin, indicating that *P. aeruginosa* pilin is not sufficient for host cell adherence (Winther-Larsen *et al*., 2007).

Although the molecular interactions involved in *P. aeruginosa* adherence remain to be more clearly defined, the mechanism of TFP-mediated adhesion has been characterized in other Gram-negative bacteria. In *N. gonorrhoeae*, a minor TFP-associated protein PilC has been identified as the adhesin due to its ability to bind eukaryotic cells and competitively block the adherence of piliated gonococci (Rudel *et al.*, 1995b). Association of PilC with gonoccocal TFP requires the pilin-like proteins PilH, PilI, PilJ, PilK, PilL, and PilV (Winther-Larsen *et al.*, 2005). PilC was initially reported to be essential for TFP biogenesis (Rudel *et al.*, 1995a, Morand *et al.*, 2004), but work by Wolfgang *et al*. (1998) demonstrated that deletion of the pilus retraction gene *pilT* could suppress the TFP biogenesis defect of a *pilC* mutant. Additionally, pilin-like proteins in both *N. gonorrhoeae* and *N. meningitidis* are required for TFP production, but this requirement can also be suppressed by inactivation of *pilT* (Winther-Larsen *et al.*, 2005, Carbonnelle *et al.*, 2006). Therefore, neither PilC, nor the pilin-like proteins, are essential for TFP biogenesis in Neisseria species, but are believed to play a role in pilus homeostasis by antagonizing PilT-dependent retraction (Wolfgang *et al.*, 2000, Morand *et al.*, 2004).

P. aeruginosa expresses a TFP-associated protein designated PilY1 that shares limited sequence homology (Alm *et al.*, 1996), but a high degree of structural similarity (Orans *et al.*, 2010) with *N. gonorrhoeae* PilC. Interestingly, the *pilY1* gene is located in an operon (*fimUpilVWXY1Y2E*) encompassing five genes that encode pilin-like proteins. A *P. aeruginosa* mutant carrying a polar insertion in *pilY1* failed to produce TFP fibers but accumulated intracellular processed pilin subunits, suggesting *pilY1* is required for fiber assembly (Alm *et al.*, 1996). Given the likely orthologous nature of PilY1 and PilC, as well as the apparent similarities between the TFP systems of *P. aeruginosa* and *N. gonorrhoeae*, we hypothesized that PilY1 mediates adherence of *P. aeruginosa* to host epithelial cells. Here, we demonstrate that PilY1 is conditionally required for TFP expression, such that TFP biogenesis is PilY1-dependent but the defect can be suppressed by inactivation of *pilT*. This discovery facilitated direct examination of the role of TFP and PilY1 in bacterial-host interactions using a well-characterized *in vitro* model of human airway epithelium. Using this model, we demonstrate that *P. aeruginosa* infection is a multifactorial process that requires TFP, pilus retraction and T3S. Further, we demonstrate the requirement for PilY1 in robust TFP-associated adherence of *P. aeruginosa* to host cell basolateral surfaces. The latter result supports a model in which TFP-associated PilY1, rather than pilin, serves as the primary adhesin for differentiated human airway epithelial cells. Overall, our findings provide a mechanistic framework for understanding the complex interactions between a

pathogen and host and the mutually dependent nature of bacterial virulence factors during infection.

Results

PilY1 is required for stable TFP production

It was previously reported that polar transposon insertions in *P. aeruginosa pilY1* resulted in a defect in TFP production (Alm *et al.*, 1996). To reassess the role of PilY1 in TFP biogenesis, we constructed a non-polar *pilY1* deletion in wild type piliated *P. aeruginosa* strain PAK and examined the ability of the wild type parent and *pilY1* mutant to produce TFP by transmission electron microscopy (TEM). Polar TFP were readily detected on the surface of the wild type strain (Fig. 1A). In contrast, the *pilY1* mutant was devoid of TFP fibers and was indistinguishable from a non-piliated *pilA* mutant (Fig. 1A). To confirm the TEM results, the wild type and mutant strains were grown on glass coverslips and pilus fibers were labeled with pilin-specific antibody and examined by immunofluoresence microscopy (IF) (Fig. 1B). While the IF technique does not provide sufficient resolution to evaluate fiber morphology, it provides an unbiased assessment of TFP production in a large population of bacterial cells. The wild type strain produced abundant TFP, while no TFP could be detected in association with either the *pilY1* or *pilA* mutants (Fig. 1B).

To further validate microscopy-based assessment of TFP production, we performed semi-quantitative analysis of TFP production by comparing the relative amount of pilin in pilus fractions recovered from the surface of the wild type and mutant strains. Since pilin is the major structural subunit of TFP, the amount of pilin recovered in pilus fractions correlates directly with TFP abundance. Pilin was recovered from the wild type strain, but was not detected in the TFP fraction recovered from the *pilY1* mutant (Fig. 2A). Comparison of the pilus preparation from the *pilY1* mutant to a serial dilution of the pilus preparation from the wild type strain indicated a greater than 100-fold reduction in TFP produced by the *pilY1* mutant (data not shown). Despite the lack of surface TFP, wild type levels of the pilin subunit were detected by immunoblot in whole bacterial cell lysates from the *pilY1* mutant (Fig. 2C) demonstrating that the TFP defect was not due to the lack of pilin.

To rule out the possibility that the TFP biogenesis defect in the *pilY1* mutant was due to a polar effect of the deletion on distal gene expression, we assessed the ability of cloned *pilY1* to restore TFP production when expressed in *trans*. A *pilY1* expression plasmid (pPa-*pilY1*) was created and transferred to the *pilY1* mutant. Induction conditions were optimized such that wild type levels of PilY1 were detected in whole bacterial cell lysates by immunoblot using PilY1-specific antiserum (Fig. 2D). Under these conditions, expression of plasmid-borne *pilY1* was sufficient to restore TFP production to a wild type level (Fig. 1 and 2A). Overall, these results provide definitive evidence that PilY1 is required for the production of *P. aeruginosa* TFP in a wild type background.

Inactivation of *pilT* suppresses the TFP production defect in a *pilY1* mutant

Inactivation of *P. aeruginosa pilT* was previously reported to results in a non-twitching, hyperpiliated phenotype in *P. aeruginosa* (Whitchurch *et al.*, 1991). In *N. gonorrhoeae*, inactivation of *pilT* suppresses the TFP biogenesis defect of a mutant lacking PilC (Wolfgang *et al.*, 1998). Given the similarities between *P. aeruginosa* PilY1 and gonococcal PilC (Alm *et al.*, 1996, Orans *et al.*, 2010), we examined the effect of inactivation of *pilT* on TFP biogenesis in the absence of PilY1. As expected, deletion of *pilT* alone resulted in a hyperpiliated, non-twitching phenotype (Fig. 1, 2A and S1). Comparison of serial dilutions of TFP containing fractions indicates that the *pilT* mutant produced approximately 4-fold more surface TFP than the wild type strain (data not shown). Deletion of *pilT* in the non-

polar *pilY1* mutant background resulted in the restoration of TFP production to a level indistinguishable from the wild type (Fig. 2A). This result demonstrates that the TFP biogenesis defect of a *pilY1* mutant is conditional and can be suppressed by inactivation of *pilT*. To confirm that the restoration of TFP production in the absence of PilY1 was due to *pilT* inactivation, we complemented the *pilY1*, *pilT* mutant with plasmid-expressed *pilT* (pPa-*pilT*). When wild type expression of PilT was restored (Fig. 2E), TFP production was abolished, resulting in a phenotype indistinguishable from that of a *pilY1* mutant (Fig. 2A and S1). Conversely, complementation of the *pilY1, pilT* mutant with plasmid-borne *pilY1* (Fig. 2D) resulted in restoration of the hyperpiliated *pilT* phenotype (Fig. 2A). Given that *pilT* is presumed to be involved in the retraction of assembled fibers, our data indicate the TFP defect seen in a *pilY1* mutant is not due to an absolute defect in TFP fiber assembly. Instead, PilY1 appears to stabilize assembled pilus fibers, a process that likely antagonizes retraction mediated by PilT. The fact that the *pilY1*, *pilT* double mutant produces less TFP than the *pilT* mutant alone (Fig. 2A), suggests that PilY1 also plays a role in stabilizing TFP assembly in the absence of fiber retraction.

Co-localization of PilY1 with TFP requires additional genes in the *pilY1*-associated operon

Given the apparent similarities between PilY1 and the gonococcal TFP-associated PilC protein, we assessed whether PilY1 is localized to the sheared pilus fraction and whether other TFP-associated proteins contribute to PilY1 localization. To assess PilY1 localization, we used PilY1-specific antiserum to probe TFP fractions recovered from the surface of the wild type and mutant strains. PilY1 was present in the wild type TFP fraction, but not detected in fractions collected from the non-piliated *pilY1* mutant or the piliated *pilY1, pilT* double mutant (Fig. 2B). Complementation of the *pilY1* and *pilY1, pilT* mutants with plasmid-encoded *pilY1* (pPa-*pilY1*) resulted in restoration of PilY1 to the TFP fractions (Fig. 2B). The TFP fraction from the non-piliated *pilA* mutant was devoid of PilY1 (Fig. 2B), despite the fact that PilY1 could be detected in whole bacterial cell lysate from the same strain (Fig. 2D). The latter result demonstrates that PilY1 localization to the TFP fraction requires the presence of surface pili. While not definitive, this result suggests that PilY1 may specifically associate with assembled pilus fibers, as is the case for gonococcal PilC (Rudel *et al.*, 1995b).

It is currently unclear if localization of PilY1 to the sheared TFP fraction requires additional proteins. However, *pilY1* is located in an operon with six additional genes (*fimU-pilVWXY1Y2E*), five of which encode pilin-like proteins with homology to *N. gonorrhoeae* proteins required for localization of PilC (Fig. 3A) (Alm *et al.*, 1997,Winther-Larsen *et al.*, 2005,Belete *et al.*, 2008). To determine if the other genes within the *pilY1* operon are required for localization of PilY1 to the sheared pilus fraction, we created an unmarked deletion encompassing the entire *pilY1* operon. The resulting mutant strain (*fimU-pilE*) was defective for TFP production (Fig. 3B). As was seen with the *pilY1* mutant, wild type levels of the pilin subunit were detected by immuoblot in whole cell lysates from the *fimU-pilE* mutant (Fig. 3D), indicating that the defect in TFP biogenesis was not due to reduced pilin availability. Complementation of the *fimU-pilE* mutant with a plasmid-borne copy of the operon (pPa-*fimU-pilE*) was sufficient to restore TFP biogenesis (Fig. 3B) and localization of PilY1 to the TFP fraction (Fig. 3C). In contrast, complementation of the *fimU-pilE* mutant with plasmid-expressed *pilY1* alone (Fig. 3E) was not sufficient to restore TFP production (Fig. 3B) or localization of PilY1 to the sheared surface fraction (Fig. 3C). While this result suggests that localization of PilY1 to surface fractions may require additional TFP biogenesis components, we cannot rule out the possibility that our fractionation technique is not sufficient for isolation of surface localized PilY1 in the absence of TFP fibers.

To determine whether other genes in the *pilY1* operon are conditionally required for TFP biogenesis, we introduced the *fimU-pilE* deletion into the *pilT* mutant background. The

resulting strain (*fimU-pilE, pilT*) produced TFP (Fig. 3B), indicating that none of the products of the *pilY1* operon are essential for TFP biogenesis. Restoration of TFP production in the absence of the pilin-like genes (*fimU-pilE*, *pilT*) provided us with the opportunity to determine the role of these proteins in PilY1 localization in an otherwise piliated background. Complementation of the *fimU-pilE*, *pilT* mutant with plasmid-expressed *pilY1* resulted in wild type levels of PilY1 in whole cell lysates (Fig. 3E); however, PilY1 was not detectable in the sheared TFP fraction (Fig. 3C) indicating that localization of PilY1 to surface TFP fractions requires both the production of TFP fibers and at least one of the pilin-like proteins encoded by the *pilY1*-associated operon.

TFP and pilus retraction are required for *P. aeruginosa* interaction with A549 cells

Previous studies have shown that TFP are required for *P. aeruginosa* adherence to mammalian cells (Doig *et al.*, 1988, Chi *et al.*, 1991). In addition, TFP-dependent host cell adherence is required for *in vivo* activation of the *P. aeruginosa* contact-dependent T3SS, which causes host cell cytotoxicity (Comolli *et al.*, 1999b, Sundin *et al.*, 2002, Wolfgang *et al.*, 2003). Although our *pilY1* mutant fails to express TFP *in vitro*, the defect appears to be at the level of fiber stabilization and not TFP biogenesis. We hypothesized that transient fiber production in the presence of the appropriate host cell receptor may be sufficient to promote host cell interaction. To assess the role of PilY1 in host cell interactions, we measured the ability of the *pilY1* mutant to adhere to and cause cytotoxicity in A549 cells (human type II pneumocyte-like carcinoma cells).

Consistent with previous reports (Chi *et al.*, 1991, Comolli *et al.*, 1999a), wild type *P. aeruginosa* adhered to A549 cells to a greater extent than the non-piliated *pilA* mutant (Fig. 4). In addition, the wild type strain was more cytotoxic than the *pilA* mutant or a *pscC* mutant, which lacks a functional T3SS (Wolfgang *et al.*, 2003) (Fig. 4). The *pilY1* mutant showed a significant reduction in both adherence and cytotoxicity compared to the wild type strain, and was indistinguishable from the *pilA* mutant (Fig. 4). Complementation of the *pilY1* mutant with plasmid-encoded *pilY1* restored both adherence and cytotoxicity.

These findings indicate that *pilY1* is required for *P. aeruginosa* to establish pathogenic interactions with host cells, but it is not clear whether the requirement is solely at the level of stable TFP production or whether PilY1 directly mediates TFP-dependent adherence. To distinguish between these possibilities, we assessed adherence and cytotoxicity of piliated *pilT* mutants in the presence or absence of *pilY1* (*pilT* versus *pilY1*, *pilT*) (Fig. 4). Both mutants had significantly reduced adherence and cytotoxicity compared to the wild type strain. While the *pilT* mutant showed greater adherence than the *pilY1, pilT* double mutant the difference was not significant. The reduced capacity of the of *pilT* mutants to adhere despite being piliated suggests that PilT, and presumably TFP retraction, is a prerequisite for interaction with A549 cells, and prevented us from drawing any conclusions about the role of PilY1 in adherence in this system.

Retractile TFP and T3S are required for infection and invasion of human ciliated airway epithelium *in vitro*

Mutants lacking the pilus retraction protein PilT were previously reported to display reduced adherence to non-polarized or transformed epithelial cell lines (Comolli *et al.*, 1999a, Sundin *et al.*, 2002). The lack of binding to A549 cells exhibited by the piliated *pilT* and *pilY1*, *pilT* mutants (Fig. 4) may be due to a defect in pilus retraction (Fig. S1), a process that is likely to facilitate tight association of *P. aeruginosa* with host cells. Experiments by Fleiszig *et al*. (1997) and Lee *et al*. (1999) demonstrated preferential binding of *P. aeruginosa* to the basolateral surfaces of well-differentiated mammalian epithelial cells. Based on these studies, we hypothesized that polarized or well-differentiated airway

epithelial cell cultures may provide a more biologically relevant model for determining the role of PilY1 in TFP-mediated adherence. To more accurately model the host environment, we evaluated *P. aeruginosa* infection in an *in vitro* model of human ciliated airway epithelium (HAE) (Fulcher *et al.*, 2005). HAE cultures are derived from freshly isolated epithelial cells from human conducting airways, which are allowed to propagate and differentiate into a pseudostratified epithelium that mimics the organization and structure of the ciliated human airway mucosal epithelium *in vivo* (Fig. 5).

To determine the feasibility of using HAE cultures for testing *P. aeruginosa* interactions with host cells, we performed initial infection experiments with wild type, piliated *P. aeruginosa.* Following apical inoculation of the HAE culture, the wild type strain rarely adhered to the ciliated mucosal surface, but by 3 hours it had invaded the epithelium and caused local infection foci (Fig. 5). By 6 hours, wild type *P. aeruginosa* spread to the basolateral compartment of the HAE cultures and appeared to interact efficiently with newly exposed basolateral surfaces of ciliated cells as well as the underlying basal epithelial cells (Fig. 5). Subsequent to invasion of the epithelium, bacterial adherence was associated with host cell rounding and detachment or extrusion. By 12 hours, the wild type infection spread to encompass the entire HAE culture. Given the efficiency with which the wild type strain was able to invade and interact with the epithelium, we expanded our study to examine the role of TFP in the infection process. In stark contrast to the wild type strain, mutants lacking TFP (*pilA*), or expressing TFP but defective for pilus retraction/twitching motility (*pilT*) did not cause invasive infection after 12 hours and could be completely removed from the intact ciliated mucosal surface of the epithelium by gentle washing (Fig. 5). Similar to the *pilA* mutant, the non-piliated *pilY1* mutant was unable to interact with the surface of the intact epithelium (data not shown). Previously published results indicate that T3S is necessary for disruption of epithelial barrier function and paracellular invasion of polarized epithelial cell monolayers (Soong *et al.*, 2008). Because *pilA* and *pilT* mutants fail to elicit T3S-dependent cytotoxicity due to their respective TFP defects (Fig. 4), we also examined the potential contribution of T3S to the invasion phenotype in the HAE model. A T3S mutant (*pscC*) was unable to invade HAE cultures and was indistinguishable from the *pilA* and *pilT* mutants (Fig. 5). These results demonstrate that invasion of the HAE is a multifactorial process that requires retractile TFP and T3S and suggests that these virulence factors are mutually dependent during infection of well-differentiated and intact tissue. The apparent lack of high affinity binding of the wild type strain or mutants to the mucosal surface of the epithelium prevented us from distinguishing between adherence and invasion in this model. However, we did observe that the *pilT* and the *pscC* mutants were capable of binding efficiently to cells that were being extruded onto the surface of the epithelium (Fig. 5). In an intact epithelium, the natural turnover of epithelial cells proceeds by extrusion of the dying cells onto the mucosal surface (Mayhew *et al.*, 1999,Pentecost *et al.*, 2006). While a relatively rare event, this observation strongly suggested that the *pilT* and *pscC* mutants were capable of binding HAE cells, despite their inability to penetrate the mucosal barrier. Our findings demonstrate that the mucosal surface of polarized and differentiated airway cells represents a substantial barrier to infection, and that once breached, *P. aeruginosa* preferentially binds to exposed basolateral surfaces.

PilY1 is required for adherence of *P. aeruginosa* to injured HAE cell cultures

The results presented above are consistent with previous studies showing that *P. aeruginosa* preferentially adheres to injured or remodeling epithelial tissue (Ramphal *et al.*, 1980, de Bentzmann *et al.*, 1996, Lee *et al.*, 1999). To determine the role of TFP and pilus-associated PilY1 in adherence to host basolateral cell surfaces, we modified the HAE cultures to artificially expose the underlying basal epithelial cells. Specifically, the columnar epithelial cells were disrupted by mechanical abrasion prior to *P. aeruginosa* infection. To assess

adherence, injured HAE cultures were exposed to bacterial strains expressing green fluorescent protein (GFP) for 45 minutes and adherent bacteria were visualized by fluorescence microscopy. Wild type *P. aeruginosa* adhered to the injured regions of the HAE, but not to the adjacent intact ciliated epithelium (Fig. 6). Similarly, the T3S mutant (*pscC*), which was unable to invade and interact with the mucosal surface of the intact epithelium (Fig. 5), showed robust adherence to the injured regions (Fig. 6), a finding that is consistent with the ability of this mutant to interact with cells extruded from the intact HAE culture. In contrast, the non-piliated *pilY1* and *pilA* mutants did not bind either injured or non-injured regions of the HAE (Fig. 6), indicting a requirement for TFP. Adherence of the *pilY1* mutant could be restored by complementation with plasmid-encoded *pilY1* (Fig. 6).

To directly examine the role of PilY1 in TFP-mediated adherence, we compared binding of the piliated non-retractile *pilT* mutant to the piliated *pilY1*, *pilT* double mutant. The *pilT* mutant, which displayed poor binding to A549 cells (Fig. 4), adhered efficiently to the injured region of the HAE (Fig. 6). Conversely, the piliated *pilY1* mutant (*pilY1*, *pilT*) mutant showed little or no binding to the injured or non-injured regions of the HAE (Fig. 6). Complementation of the *pilY1*, *pilT* mutant with plasmid-expressed *pilY1* restored adherence (Fig. 6). To determine whether localization of PilY1 to the pilus fraction is necessary for adherence, we assessed binding of the piliated *fimU-pilE*, *pilT* mutant and found it was unable to bind the injured epithelium (Fig. 6). Complementation with the entire *pilY1*-associated operon (pPa-*fimU-pilE*), but not *pilY1* alone (pPa-*pilY1*) restored adherence (Fig. 6). These results demonstrate that the adherence of *P. aeruginosa* to injured HAE cultures requires PilY1 localization to the surface TFP fraction and strongly suggest that PilY1 functions as a TFP-associated adhesin. Further, the inability of the piliated, PilY1-lacking strain (*pilY1*, *pilT*) to adhere to host cells indicates that pilin, in its polymerized form, is not sufficient for adherence to well-differentiated human airway epithelial cells.

To confirm the results of the injured HAE binding experiments, we directly visualized bacterial-host cell interactions in fixed histological sections of the injured HAE regions by light microscopy. In all cases, the bacterial strains that adhered to the injured regions of the HAE were specifically associated with exposed basal epithelial cells (Fig. 7). In contrast, the non-adherent mutants (*pilA*, *pilY1* and *pilY1*, *pilT*) were rarely detected in association with exposed underlying host cell surfaces (Fig. 7). Interestingly, adherent strains that retained cytotoxicity (T3S) and twitching motility (Fig. 4 and S1; wild type and the complemented *pilY1* mutant) appeared to further disrupt the integrity of the basal cell layer and penetrated to the level of the culture membrane support during the 45 minute infection (Fig. 7). In contrast, the adherent twitching-defective strains (Fig. S1; *pilT* and *pilY1*, *pilT* complemented with plasmid-expressed *pilY1*) did not further disrupt the integrity of or penetrate the exposed basal cell layer (Fig. 7). In addition, the non-cytotoxic T3S mutant (*pscC*) interacted with the superficial exposed or damaged basal cells but did not penetrate further into the exposed basal cell layers (Fig. 7). These result indicates that although binding of *P. aeruginosa* to exposed basal cells does not require pilus retraction or T3S, subsequent intercellular spread within the damaged tissue depends on twitching motility and the ability to deliver toxic effector proteins.

Discussion

Adherence to and infection of mucosal epithelium is a crucial event in the pathogenesis of human disease caused by the opportunistic pathogen *P. aeruginosa.* However, the factors contributing to this event have yet to be clearly defined. Here, we examined the role of the PilY1 protein and pilus retraction in TFP-mediated adherence using a genetic approach. Initially, we examined the involvement *P. aeruginosa* PilY1 in TFP biogenesis and function. We found that PilY1 is not a canonical pilus assembly factor but rather acts as a conditional

effector of pilus homeostasis by promoting extension/polymerization events in the presence of the pilus retraction ATPase PilT, a function previously demonstrated for the related Neisseria PilC proteins (Wolfgang *et al.*, 1998, Carbonnelle *et al.*, 2006). In addition, we confirmed that PilY1 co-purifies with TFP in *P. aeruginosa* and further showed that this localization requires at least one of a set of genes linked to and co-transcribed with *pilY1* (*fimU-pilVWXY1Y2E*). As previously noted, the *fimU* operon displays synteny with the *N. gonorrhoeae pilH-L* locus whose products are necessary for localization of PilC to the TFP fraction (Winther-Larsen *et al.*, 2005). In contrast to our results and previous results (Alm *et al.*, 1996), Bohn and colleagues (2009) detected a truncated form of PilY1 in concentrated *P. aeruginosa* culture supernatants but were unable to detect full-length PilY1 in extracellular fractions by immunnoblot or in association with TFP fibers by immunogold labeling and electron microscopy. Technical differences between the studies, including the fractionation methods employed and the reagents and bacterial strains used likely account for the discrepancy.

Transcription of the *fimU-pilVWXY1Y2E* operon, but not the *pilA* gene (encoding pilin), is controlled by intracellular cyclic AMP in combination with the cyclic AMP receptor protein Vfr and the AlgZ/AlgR two component regulatory system (Lizewski *et al.*, 2002, Wolfgang *et al.*, 2003, Belete *et al.*, 2008), suggesting that the level of PilY1 and associated proteins can be modulated independent of the rest of the TFP biogenesis machinery. This circuitry may allow *P. aeruginosa* to alter PilY1-dependent TFP production and associated phenotypes (twitching motility and host cell adherence) in response to environmental conditions. Regulation of *pilY1* expression may be analogous to *pilC* regulation in *N. meningitidis* where host cell contact results in reduced *pilC* transcription (Taha *et al.*, 1998, Morand *et al.*, 2004). Interestingly, it has been reported that deletion of *pilY1* results in the altered expression of numerous *P. aeruginosa* genes including activation of *lipC*, encoding a secreted lipase and the *fimU-pilVWXY1Y2E* operon (Martinez *et al.*, 1999, Bohn *et al.*, 2009). Furthermore, it was shown that *pilY1* mutant strains have increased autolysis and reduced secretion of secondary metabolites including the redox-activated pigment pyocyanin and 4-hydroxy-2-alkylquinolines (Bohn *et al.*, 2009). These results suggest that the expression and/or localization of PilY1 may serves as a feedback signal for altering gene expression. While it remains to be determined whether pilus retraction or bacterial attachment elicit the same response as *pilY1* deletion, it is conceivable that these mechanical processes result in the altered localization of PilY1, which in turn acts as a signal for changes in gene expression and/or production and secretion of virulence factors and toxic secondary metabolites.

Next, we evaluated the role of PilY1 in *P. aeruginosa* adherence to human cells by using a piliated non-retractile *pilT* mutant background in which TFP expression was maintained in the absence of PilY1. In our studies with the A549 carcinoma cell line, the piliated *pilT* and *pilY1, pilT* mutants showed significantly reduced adherence compared to the wild type strain, which precluded any conclusions regarding the role of PilY1 in adherence. Similarly, the piliated *pilT* and *pilY1, pilT* mutants were unable to interact with the mucosal surface of intact HAE cultures or cause invasive infection. However, injury of the HAE cultures supported robust adherence of the *pilT* mutant to regions of exposed basal cells. The fact that the piliated *pilY1, pilT* mutant, the piliated *fimU-pilE, pilT* mutant and the piliated *fimU-pilE, pilT* mutant complemented with plasmid-expressed *pilY1* (in which PilY1 does not localize with TFP) all failed to bind the injured HAE cultures provides genetic evidence that PilY1 serves as a TFP-associated adherence factor in this model. Although these results do not formally rule out the contribution of an integral adhesive component in pilin, they do question the relevance of such an activity as it is unclear why it would only be manifest in conjunction with PilY1 expression.

Our results suggest that bacterial adherence to non-polarized transformed cell line and injured HAE cultures involve different adhesin-receptor interactions. We hypothesize that the interaction between *P. aeruginosa* and primary well-differentiated human airway epithelial cells more accurately reflects the *in vivo* situation. The ability to *P. aeruginosa* to recognize a high affinity/abundant receptor on the basolateral surfaces of HAE cells is consistent with previous work showing that *P. aeruginosa* preferentially infects polarized epithelium via a basolateral route (Fleiszig *et al.*, 1997, Lee *et al.*, 1999). The involvement of distinct adhesin-receptor interactions is supported by our observation that the piliated non-retractile *pilT* mutant bound to the basolateral surface of primary epithelial cells but not to A549 cells. This result suggests that pilus retraction may increase the affinity of an adhesin-receptor interaction during attachment to non-polarized transformed cells. An analogous scenario has been described for *Escherichia coli* lectin-like adhesin FimH, where shear forces dramatically increase adhesin-receptor affinity by a catch-bond mechanism (Thomas *et al.*, 2002). Alternatively, pilus retraction may facilitate additional bacterial-host cell interactions necessary to support adherence to transformed cells, similar to the cascade of adherence events that occur during colonization of intestinal epithelial cells by enteropathogenic *E. coli* (EPEC). Initial or localized adherence of EPEC involves bacterial attachment to an *N*-acetyllactosamine contain receptor via α-bundlin, the major subunit of bundle-forming pili (Hyland *et al.*, 2008). Following localized adherence, EPEC established intimate attachment, a process mediated by intimin, an EPEC outer membrane protein that specifically binds the Tir receptor, a bacterial protein delivered to host cells by the EPEC T3SS (Nougayrede *et al.*, 2003, Cleary *et al.*, 2004).

Our HAE culture studies indicate that *P. aeruginosa* gains access to the basolateral compartment following inoculation of the apical surface of the intact epithelium (Fig. 5). Pathogenic bacteria have evolved a number of discrete mechanisms to gain access to these privileged basolateral sites including invading M cells in order to traverse the epithelium or by disrupting the integrity of epithelial cell tight junctions (Jensen *et al.*, 1998,Soong *et al.*, 2008,van Alphen *et al.*, 2008). In the case of *Listeria monocytogenes*, the bacteria appear to invade epithelial junctions at sites of cell extrusion and expulsion occurring during epithelial renewal (Pentecost *et al.*, 2006). Such an event results in the transient exposure of basolateral cell surfaces and components (Pentecost *et al.*, 2006) and may represent a similar route used by *P. aeruginosa* to breach the epithelial barrier. Alternatively, it has been proposed that *P. aeruginosa*-induced tissue damage alters the *N*-glycoprotein and heparan sulfate proteoglycan receptor composition of the apical and basolateral compartments, thereby allowing bacterial access to basolateral cell surfaces via receptor-mediated bacterial binding (Bucior *et al.*, 2010). Once the epithelial barrier has been bypassed, we hypothesize that interaction between *P. aeruginosa* and components present on host basolateral membranes facilitates intercellular spread of the bacteria, similar to the basolateral-specific invasion mechanisms used by *L. monocytogenes*, *Shigella flexneri*, and *Campylobacter jejuni* (Mounier *et al.*, 1992,Gaillard *et al.*, 1996,Monteville *et al.*, 2002). Further, our observation that *P. aeruginosa* preferentially adheres to mechanically injured epithelial tissue *in vitro* (Fig. 6) is consistent with studies showing “opportunistic adherence” to damaged areas of mammalian tissue (Ramphal *et al.*, 1980,de Bentzmann *et al.*, 1996,Lee *et al.*, 1999) and provides a mechanistic explanation for the increased rate of *P. aeruginosa* infection following tissue damage (wounds, corneal abrasion, burns, surgery and endotracheal intubation).

Based on our studies with intact and damaged HAE cultures, we propose a sequence of events leading to fulminant tissue infection by *P. aeruginosa* (Fig. 8). We hypothesize that *P. aeruginosa* infection of intact mucosal epithelium can be initiated by a relatively rare adherence event (Fig. 8A) that evolves into basolateral infection (Fig. 8B and C). While we were unable to detect the earliest adherence events preceding tissue invasion by wild type *P.*

aeruginosa, we did capture these events with the *pilT* and *pscC* mutants. Both the piliated non-retractile *pilT* mutant and the piliated but non-cytotoxic *pscC* mutant bound to epithelial cells that had been extruded onto the surface of the HAE cultures or were undergoing extrusion from the otherwise intact epithelium (Fig. 5). Although the *pilT* and *pscC* mutants adhered to extruded cells, these strains were unable to penetrate the mucosal barrier to cause invasive infection, indicating an early block in the infection process. Given that non-piliated mutant strains (*pilA* and *pilY1*) were unable to bind mechanically exposed basal cells (Fig. 6 and 7) and that adherence of the *pilT* mutant to exposed basal cells required TFP-associated PilY1 (Fig. 6 and 7), we propose that TFP and TFP-associated PilY1 are required for initial binding to the mucosal surface at the site of cell extrusion. Subsequent interactions between *P. aeruginosa* and the intact apical surface lead to a breach of the epithelial barrier. This process likely involves T3S-dependent alteration of tight junctions (Soong *et al.*, 2008) or the relocalization of apical and basolateral receptors in response to bacterial-induced host cell damage (Bucior *et al.*, 2010). Regardless of the initiating mechanism for apical penetration, we hypothesize that subsequent access of *P. aeruginosa* to normally masked basolateral receptors allows attachment and bacteria-induced cytotoxicity requiring PilT-dependent pilus retraction and T3S (Fig. 8B). Assuming the requirement for PilT reflects its role in TFP retraction (Merz *et al.*, 2000), two independent functions can be envisioned: 1) pilus retraction brings the bacteria into intimate contact with host cells and thus facilitates activation of the contact-dependent T3SS and efficient effector delivery, and/or 2) pilus retraction promotes intercellular spread of the bacteria via twitching motility. Further dissemination of the bacteria within the basal compartment of the stratified tissue (Fig. 8C) requires PilY1, PilT and T3S such that bacterial adherence, tissue damage and dissemination mutually fuel a positive feedback loop that culminates in fulminant infection.

In summary, we have identified and characterized a novel, multifactorial strategy by which *P. aeruginosa* gains access to and damages human mucosal tissue. In addition, this study provides compelling evidence that TFP-associated PilY1 is an adhesin that specifically recognizes a basolateral host cell receptor. Further biochemical and structural studies of PilY1 should reveal how it exerts its adherence-promoting effect and undoubtedly facilitate the identification of the host ligand(s) involved.

Experimental Procedures

Bacterial Strains and Growth Conditions

All *P. aeruginosa* strains and plasmids used in this study are described in Table S1. Unmarked, non-polar deletion alleles were constructed as previously described (Wolfgang *et al.*, 2003) using the oligonucleotides listed in Table S2. The *pilY1* deletion allele carries an in-frame stop codon (TGA) in place of the nucleotides encoding amino acid residues 51 through 801 of the strain PAK *pilY1* coding sequence (GenBank #EU234515). The *fimU-pilE* deletion was engineered to remove the entire *pilY1* operon (*fimUpilVWXY1Y2E*) sequence between the *fimU* start codon (ATG) to the *pilE* stop codon (TGA). Deletion alleles were introduced onto the chromosome of *P. aeruginosa* strain PAK as described (Wolfgang *et al.*, 2003) and confirmed by PCR and DNA sequencing. *P. aeruginosa* strains were routinely grown at 37°C in Luria Bertani (LB) broth or on LB agar. The pMMB-based expression plasmids were maintained in *P. aeruginosa* with 150 μg ml^{-1} carbenicillin (Cb), except where noted. Bacterial growth in broth culture was assessed by optical density at 600 nm (OD_{600}). Constitutive GFP expression was achieved by transferring plasmid pSMC21 into wild type and mutant *P. aeruginosa* strains as previously described (Bloemberg *et al.*, 1997).

Expression Plasmid Construction

The open reading frames (ORFs) of *pilY1* and *pilT* and the entire *pilY1*-associated operon (*fimUpilVWXY1Y2E*) were amplified from strain PAK chromosomal DNA and cloned into the expression plasmid pMMBV2GW as described previously (Wolfgang *et al.*, 2003), to yield pPa-*pilY1*, pPa-*pilT* and pPa-*fimU-pilE*, respectively. Oligonucleotide primers used to generate expression plasmids are indicated (Table S2). Plasmid pMMBV2GW was generated from the broad host-range expression plasmid pMMBGW (Wolfgang *et al.*, 2003) by changing the sequence of the −35 and −10 regions of the *tac* promoter from TTGACA to TTTACA and from TATAAT to CATTAT, respectively (S. Lory, Harvard Medical School, unpublished). The resulting *tac* promoter modifications result in tighter transcriptional repression of cloned down-stream genes in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG) induction (data not shown). All expression plasmids were transferred to the appropriate strain by conjugation (Furste *et al.*, 1986) followed by selection on LB agar plates containing 150 μg ml^{-1} Cb and 25 μg ml^{-1} irgasan. Following selection, expression plasmid carrying strains were grown in the presence of 30 μg ml^{-1} Cb. To achieve wild type expression of the cloned genes, strains containing expression plasmids were grown in the presence of 20 (pPa-*pilT)* or 75 μM IPTG (pPa-*pilY1 and* pPa-*fimU-pilE*).

Electron and Immunoflourescence Microscopy

Transmission electron microscopy (TEM) was performed as described previously (Winther-Larsen *et al.*, 2007), with the exception that grids were placed on a drop of bacterial suspension at 22°C for 10 minutes and the samples were stained with an aqueous 0.5% ammonium molybdate solution for 10 minutes. Immunofluorescence (IF) microscopy was performed as described previously (Winther-Larsen *et al.*, 2005), except that the *P. aeruginosa* strains were grown until OD_{600} = 0.2 prior to incubating the bacteria on poly-L-lysine-coated glass cover slips. *P. aeruginosa* pilin-specific antiserum (gift of E. C. Gotschlich, Rockefeller University) was used as a primary antibody for TFP labeling, followed by an Alexa Red 594-conjugated goat anti-rabbit IgG (Molecular Probes). *P. aeruginosa* cells were strained with 4′-6-diamidino-2-phenylindole (DAPI) at 1 μg ml^{-1} in Mowiol Mounting Medium (Sigma) containing 2% 1,4-diazabicyclo(2,2,2)octane (DABCO) prior to viewing with a Nikon Eclipse C400 fluorescence microscope.

Pilus Purification

P. aeruginosa strains were growth on LB agar plates; plasmid-harboring strains were grown on plates agar containing 30 μg ml^{-1} Cb and the indicated amount of IPTG. After incubation at 37°C for 18 hours, bacteria were collected and suspended in 10 ml of 0.15 M NaCl and 0.2% formaldehyde. The suspensions were vortexed for 1 minute to release surface TFP and bacterial cells were removed by centrifugation at 12,000 × *g* for 5 minutes. Supernatants were transferred to 15 ml glass Corex tubes, adjusted to 0.1 M $MgCl_2$, and incubated at 4°C for 3 hours. Following centrifugation 12,000 × *g* for 5 minutes, the resulting TFP pellets were washed and suspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, resolved on an 18% polyacrylamide gel and visualized by GelCode Blue Stain (Pierce).

Twitching Motility Assay

P. aeruginosa strains were stab-inoculated to the bottom of 100 mm tissue culture-treated dishes (Corning) containing 5 ml LB plus 1% agar. For assays with plasmid-harboring strains, bacteria were grown in the presence of 30 μg ml^{-1} Cb and the indicated amount of IPTG. Plates were incubated for 48 hours at 37°C in a humidified chamber and the zone of subsurface bacterial growth radiating from the point of inoculation was measured.

Immunoblotting

Whole cell lysates were prepared from bacteria grown in LB broth to mid-exponential growth phase (OD_{600} = 1). Bacteria were collected by centrifugation, suspended in 50 μl of SDS-PAGE sample buffer and total cellular proteins were separated by SDS-polyacrylamide gels and transferred to nitrocellulose. Membranes were probed with the following primary antibodies: PKL1 anti-pilin mouse monoclonal antibody (Yu *et al.*, 1994) (1:30,000 dilution, gift of Randall Irvin, University of Alberta), anti-PilT rabbit serum (1:30,000 dilution, gift of Katrina Forest, University of Wisconsin), or anti-PilY1 rabbit serum (1:4000 dilution) generated against a purified C-terminal portion of PilY1. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (for PilT and PilY1) and goat anti-mouse IgG (for pilin) were used as secondary antibodies. Blots were developed with enhanced chemiluminescence reagents (Millipore) and visualized by autoradiography.

A549 Bacterial Adherence and Cytotoxicity Assays

Routine passage of A549 cells was performed as previously described (Ichikawa *et al.*, 2005). For bacterial challenge assays, A549 cells were seeded and grown on sterile glass coverslips placed in 6-well tissue culture plates (adherence assays) or in 24-well tissue culture plates (cytotoxicity assays). For adherence assays, bacteria were grown to mid-log growth phase in LB broth and washed and diluted in Hank's Balanced Salt Solution (HBSS) plus supplements (1 mM $CaCl_2$, 2 mM $MgCl_2$ and 20 mM HEPES). Bacteria (2 ml) were introduced at a multiplicity of infection (MOI) of 50 bacteria per A549 cell and the infection was allowed to proceed for 1 hour at 37°C in 5% CO_2. The A549 cells were washed 3 times with 1 ml of HBSS plus supplements to remove unattached bacteria. The co-cultures were fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for 1 hour and then in 5% formaldehyde, 5% glacial acetic acid and 70% methanol for 1 hour, followed by staining with Giemsa stain for 10 minutes. Coverslips were mounted on glass slides, viewed by light microscopy at 100 X magnification and the eukaryotic cells and associated bacteria from 6 fields, representing different regions of the coverslip, were enumerated and reported as number of bacteria per A549 cell. Cytotoxicity assays were performed using bacteria grown to mid-log phase in LB broth and washed in Dulbecco's Modified Eagle's Medium. A549 cells were infected with bacteria (500 μl) at an MOI of 10 for 10 hours at 37°C in 5% CO_2. Cytotoxicity was assessed by measuring host cell lactate dehydrogenase (LDH) release using a commercially available LDH assay kit (Takara Bio). LDH release from infected cultures was reported relative to the percent of LDH released from A549 cells treated with 0.25% Triton X-100.

HAE Cultures and *P. aeruginosa* Infection and Binding Assays

Human airway tracheobronchial epithelial cells were isolated from excess tissue following lung transplantation under University of North Carolina at Chapel Hill Institutional Review Board-approved protocols by the UNC Cystic Fibrosis Center Tissue Procurement and Cell Culture Core. Primary cells were expanded on plastic and plated on permeable Transwell-Clear membrane supports (Corning, Inc.). HAE cultures were grown in custom media (ALI) with provision of an air-liquid interface for 4 to 6 weeks to form differentiated, polarized cultures that resemble *in vivo* pseudostratified mucociliary epithelium, as previously described (Fulcher *et al.*, 2005). HAE cultures were transferred to antibiotic-free media 1 day prior to bacterial infection. For infection of intact HAE cultures, wild type and mutant *P. aeruginosa* strains were grown to mid-exponential growth phase (OD_{600} = 1.0), washed twice with sterile PBS and suspended in antibiotic-free ALI at a concentration of 4×10^7 CFU ml^{-1}. For each strain, 25 μl of bacterial suspension was added to the mucosal surface of the HAE cultures at an approximate MOI of 10. Infected cultures were incubated at 37°C and 5% CO_2 for 3, 6 or 12 hours, washed twice with pre-warmed antibiotic-free ALI and then fixed in a solution of 2% formaldehyde, 2% paraformaldehyde overnight. For infection of injured HAE cultures, the cultures were damaged by lightly scraping a sterile pipette tip

across the mucosal surface to remove ciliated columnar epithelial cells. GFP-expressing *P. aeruginosa* strains were grown in the presence of 50 μg ml^{-1} kanamycin (to ensure maintenance of plasmid pSCM21), washed twice with sterile PBS and suspended in antibiotic-free ALI to a concentration of 4×10^8 CFU ml^{-1}. Bacterial suspensions (250 μl) were added to the apical surface of the injured HAE cultures at an approximate MOI of 1000. After incubation at 37°C and 5% CO_2 for 45 minutes, HAE cultures were washed six times with pre-warmed antibiotic-free ALI. After the final wash, all media was removed from the surface of the HAE cultures. Binding of GFP *P. aeruginosa* to the injury site was recorded using a Leica DMIRB Inverted Fluorescence/DIC microscope with a black/white digital camera. Phase contrast images of the infected cultures were taken in parallel to locate the site of injury. After imaging, infected HAE cultures were fixed in a solution of 2% formaldehyde, 2% paraformaldehyde overnight. Fixed HAE cultures, (intact and injured) were embedded in epon resin and sectioned for histological examination. Semi-thin sections were stained with Richardson's stain to allow identification of single bacteria and HAE cells. Representative sections were visualized by light microscopy and imaged with a digital camera.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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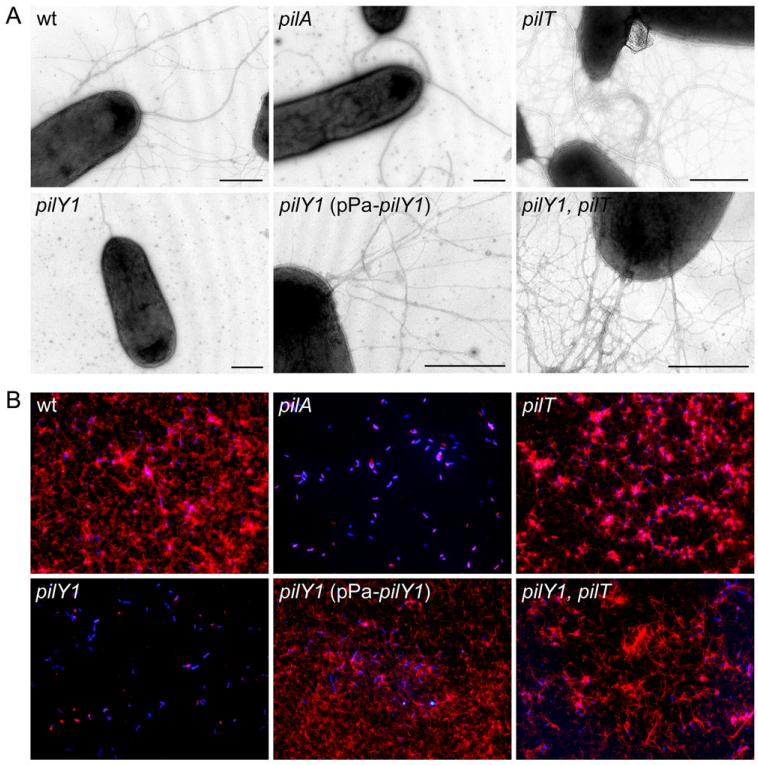

Fig. 1. PilY1 is conditionally required for TFP production

Surface TFP were visualized by (A) transmission electron microscopy and (B) immunofluorescence microscopy. Pilus fibers (thin) are visible on the surface of the wild type (wt) strain and a non-retractile *pilT* mutant. In contrast, a *pilY1* mutant is devoid of TFP and indistinguishable from a non-piliated control strain (*pilA*). Wild type levels of TFP are restored in a *pilY1* mutant complemented with plasmid-expressed *pilY1* (pPa-*pilY1*). Pilus fibers are also restored in a *pilY1* mutant when *pilT* is inactivated (*pilY1*, *pilT*), indicating PilY1 is not an essential TFP biogenesis factor. Strain genotype is indicated in each panel. For electron microscopy, scale bars equal 500 nm. Thick fibers visible on the wt strain and *pilA*, *pilT* and *pilY1* mutants are flagella. For immunofluorescence, bacterial cells were labeled with DAPI (blue) and TFP were labeled with pilin-specific antibody, Alexa Red 594-conjugated goat anti-rabbit secondary antibody (red) and bacteria were imaged at 100X magnification.

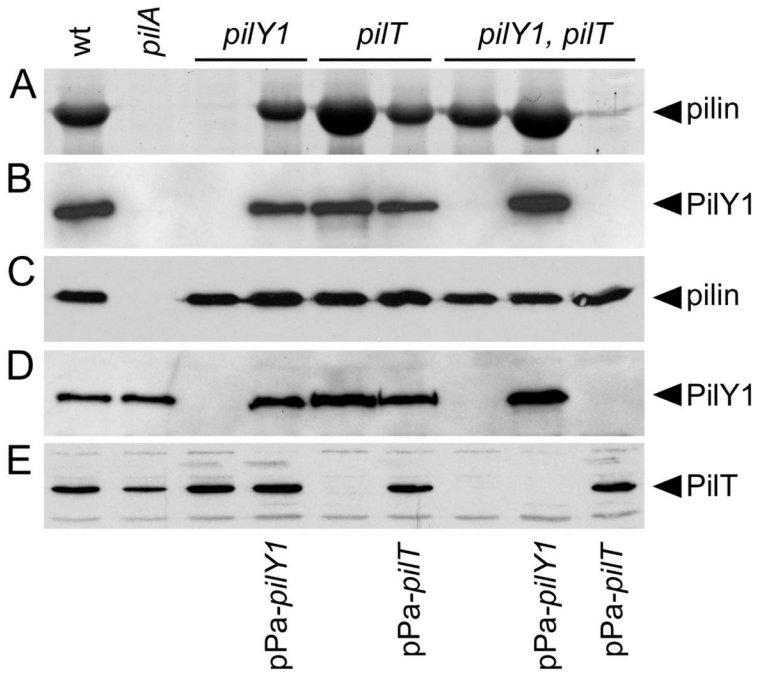

Fig. 2. PilY1 is conditionally required for TFP production and co-localizes with sheared surface fibers

(A) Coomassie Blue-stained SDS-PAGE gel showing the relative abundance of recovered pilin in sheared pilus fractions. (B) Immunoblot of pilus fractions probed with PilY1-specific antiserum. (C-E) Immunoblots of whole bacterial cell lysates separated by SDS-PAGE and probed with pilin-specific (C), PilY1-specific (D) or PilT-specific (E) antiserum.

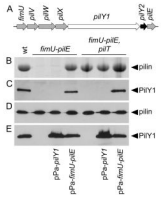

Fig. 3. Localization of PilY1 to the pilus fraction requires proteins encoded by the *pilY1*-associated operon

(A) Organization of the *pilY1*-associated operon. Pilin-like genes are shaded grey. (B) Coomassie Blue-stained SDS-PAGE gel showing the relative abundance of recovered pilin in pilus fractions. (C) Immunoblot of surface pilus fractions probed with PilY1-specific antiserum. (D-E) Whole bacterial cell lysates separated by SDS-PAGE and probed with either pilin-specific (D) or PilY1-specific (E) antiserum by immunoblotting.

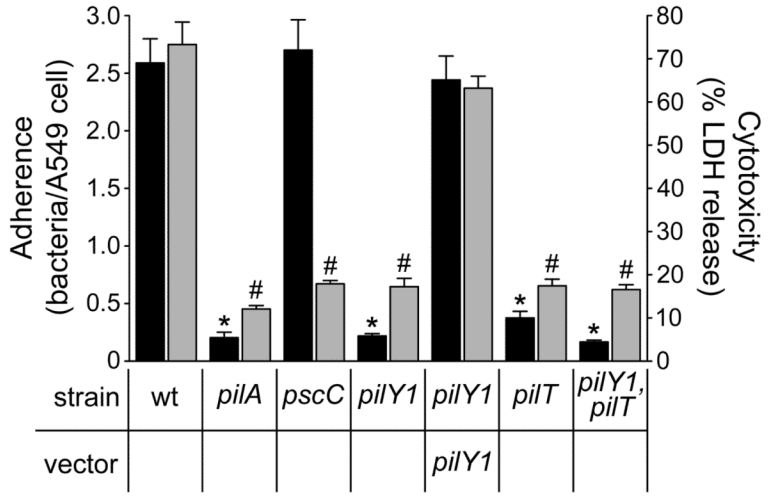

Fig. 4. Retractile TFP and pilus-associated PilY1 are necessary for productive interactions with A549 epithelial cells

Bacterial adherence (black bars; mean +/− SEM; *n*=5) and cytotoxicity (grey bars; mean +/− SEM; *n*=3) to A549 cells is shown. Bacterial adherence was determined based on the average number of bacteria bound per A549 cell. Adherence to A549 cells was significantly reduced (*; p<0.001) in the non-pilated *pilA* and *pilY1* mutants and in the piliated non-retractile *pilT* and *pilY1*, *pilT* mutants compared to wild type. Cytotoxicity was determined based on the percentage (%) of lactate dehydrogenase (LDH) released from A549 cells following bacterial infection relative to the amount of LDH released following cell lysis with 0.25% Triton X-100. The ability to elicit a cytotoxic effect was significantly reduced (#; p<0.001) in the non-adherent strains and in a T3S mutant (*pscC*) compared to wild type.

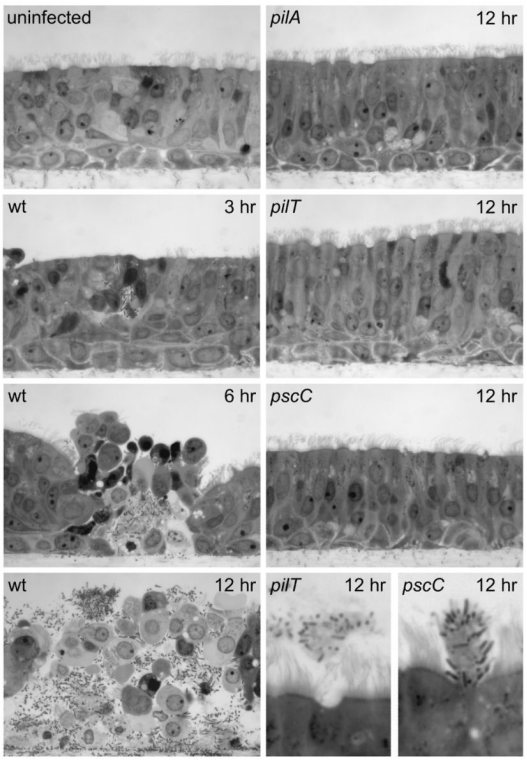

Fig. 5. Invasive infection of Human Airway Epithelium (HAE) cultures requires retractile TFP and Type III Secretion

Histological cross-sections of HAE cultures inoculated with wild type (wt), *pilA*, *pilT* and *pscC* strains. Representative images taken at 3, 6, and 12 hours post-infection are shown. Wild type *P. aeruginosa* rarely adhered to the ciliated mucosal surfaces but local infection foci could be detected after 3 hours. After breaching the mucosal barrier, the wild type strain interacted efficiently with the basolateral surfaces of ciliated cells and the underlying basal epithelial cells. Bacterial adherence was associated with host cell rounding and detachment. By 12 hours, the wild type infection appeared to spread between cells to encompass the entire HAE culture. In contrast, the non-piliated *pilA* mutant, the piliated non-retractile *pilT* mutant and the T3S mutant (*pscC*) did not interact with the epithelium or cause invasive infection. However, unlike the *pilA* mutant, the *pilT* and *pscC* mutants retained the ability to interact with shed or extruding epithelial cells (bottom right panels).

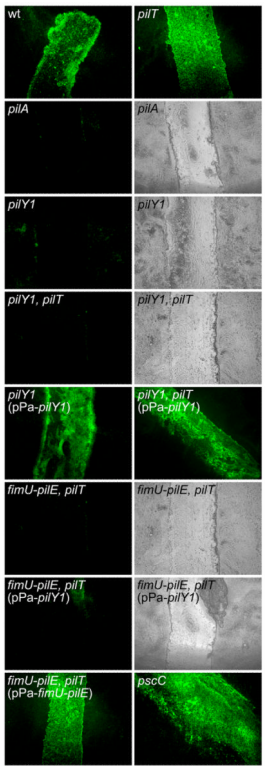

Fig. 6. TFP and pilus-associated PilY1 are required for adherence of *P. aeruginosa* to injured HAE cultures

Injured HAE cultures were imaged (en face) by fluorescence microscopy 45 minutes after inoculation with bacterial strains expressing GFP. The wild type (wt) strain, the piliated *pilT* mutant and a non-cytotoxic T3S mutant (*pscC*) attached efficiently to the injured tissue but showed limited interaction with the adjacent intact epithelium. The non-piliated (*pilY1*) and piliated (*pilY1*, *pilT*) *pilY1* mutants did not adhere to the injured tissue and were indistinguishable from the non-piliated control mutant (*pilA*). Adherence was restored for both the non-piliated and piliated *pilY1* mutants following complementation with plasmid-expressed PilY1 (pPa-*pilY1*). The piliated *fimU-pilE, pilT* mutant was unable to adhere to the damaged tissue; complementation with the entire *fimU* operon (pPa-*fimU-pilE*) but not *pilY1* alone (pPa-*pilY1*) restored adherence. For non-adherent strains, tissue damage was confirmed by examining the HAE cultures by both fluorescence and light microscopy.

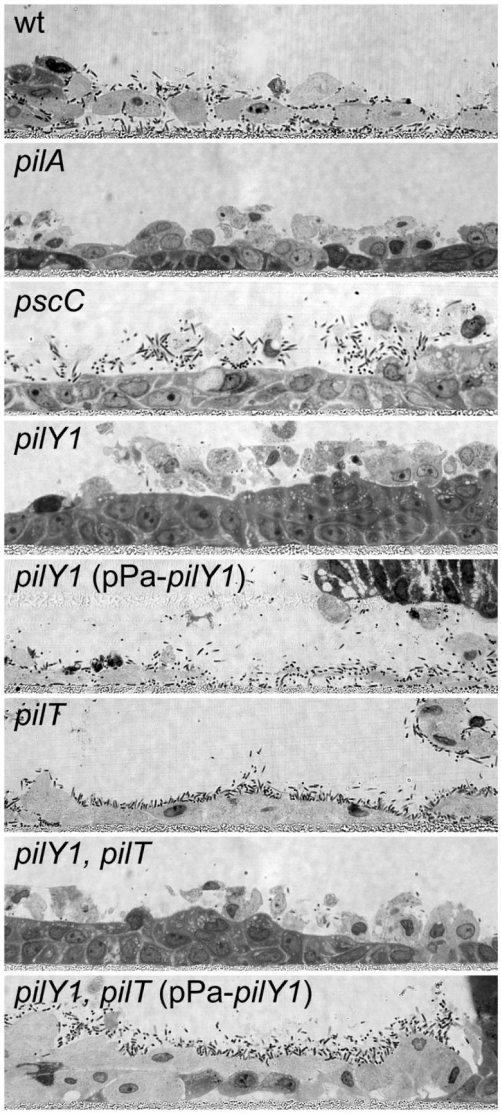

Fig. 7. PilY1 is required for adherence to exposed basal epithelial cells of injured HAE cultures

Histological sections of HAE cultures show the presence of bacteria on newly exposed basal epithelial cells after injury. The wild type strain (wt), the piliated *pilT* mutant and a non-cytotoxic T3S mutant (*pscC*) showed robust adherence, while the non-piliated (*pilY1*) and piliated (*pilY1*, *pilT*) PilY1-lacking strains were defective for binding to basal epithelial cells at the injury site. Adherence was restored for both the non-piliated and piliated *pilY1* mutants following complementation with plasmid-expressed *pilY1* (pPa-*pilY1*). Only strains which retained twitching motility and cytotoxicity (wt and the complemented *pilY1* mutant) were able to further penetrate the exposed basal cell layers.

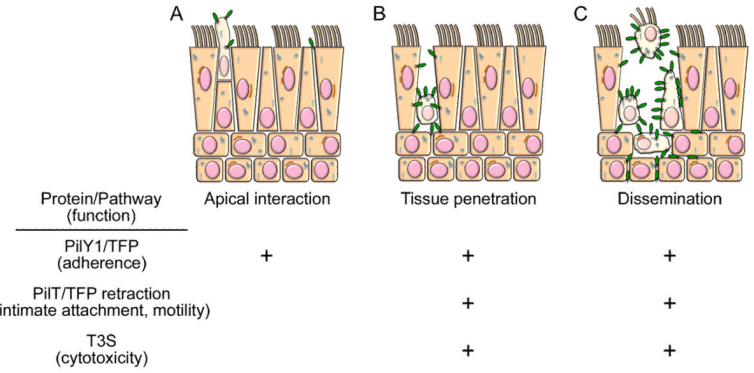

Fig. 8. Model of events leading to invasive infection of the human airway epithelium by *P. aeruginosa*

The three different phases of epithelial infection (apical interaction, penetration and dissemination) and the *P. aeruginosa* factors required for each phase are indicated. (A) **Apical interaction**. Productive interactions between *P. aeruginosa* and the intact mucosal epithelium is a relatively rare event exploiting transiently exposed basolateral surfaces during cell extrusion. This event is dependent on bacterial adherence mediated by TFP and TFP-associated PilY1. (B) **Penetration**. Subsequent penetration of the mucosal barrier requires retractile TFP and T3S. PilT-dependent pilus retraction may facilitate contact-dependent T3S and TFP-mediated bacterial motility. Cytotoxicity mediated by T3S causes additional tissue damage and disruption of host cell junctions and exposure of additional basolateral host receptors. (C) **Dissemination**. Following the formation of a focal infection, PilY1-mediated adherence, retractile TFP and T3S act synergistically to cause fulminant infection and dissemination into deeper tissue.