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Pseudomonas aeruginosa injects type III effector ExoS into epithelial cells through the function of type IV pili

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ABSTRACT

Translocation of *Pseudomonas aeruginosa* through epithelial tissues can cause sepsis. Here, we examined whether *P. aeruginosa* penetrates epithelial cell layers using type IV pili (TFP). Deletion of TFP (*pilA*) did not affect association with Caco-2 cells, although it decreased penetration through, and disruption of, Caco-2 cell monolayers. We found that TFP are necessary for injection of the type III effector ExoS, which impairs defense against *P. aeruginosa* penetration, into host cells. Deletion of *pilA* attenuated oral infection in silkworms. We conclude that *P. aeruginosa* injects ExoS into cells through the function of TFP, enabling penetration of epithelial barriers.

1. Introduction

Pseudomonas aeruginosa is a major opportunistic pathogen that causes pneumonia and bacteremia, both of which are associated with high mortality rates and are often difficult to treat in immunocompromised patients [1,2]. *P. aeruginosa* colonization of the respiratory, urinary and gastrointestinal tracts in immunocompromised patients can trigger a wide range of severe acute and chronic complications in intensive care units (ICUs) [3]. In a hospital, typical environmental reservoirs include basins, showers and the rinsing water of manually prepared bronchoscopes [4]. In addition, the gastrointestinal tract is considered an endogenous reservoir for *P. aeruginosa* in immunocompromised patients, and translocation of the colonized *P. aeruginosa* from the gastrointestinal tract can cause severe bacteremia leading to fatal sepsis [3–7]. Additionally, in vivo experiments using neutropenic mice highlighted the importance of the translocation of *P. aeruginosa* from the colonized gastrointestinal tract in cases of severe sepsis [8,9]. Collectively, these reports suggest that the elucidation of the translocation mechanisms of *P. aeruginosa* from the gastrointestinal tract is required for a better understanding of *P. aeruginosa* opportunistic infections.

To translocate to the blood from a host lumen, bacteria must cross two major barriers, the mucous and epithelial cell layers [10–12]. Epithelial cells, including those from the gastrointestinal, respiratory and urinary tracts, attach to adjacent cells via tight junctions, adherens junctions and desmosomes [13]. Tight junctions provide barriers to prevent leakage of molecules, including integral proteins and lipids, across the epithelia through the gaps between cells [13]. Three distinct types of transmembrane proteins have been localized to tight junctions; occludin [14], claudins [15], and junctional adhesion molecules [16]. The lethal effects of intestinal *P. aeruginosa* are dependent on its ability to adhere to and disrupt epithelial cell layers [17–19]. We consider that penetration of *P. aeruginosa* through the epithelial cell layer is at least a five-step process involving: (i) recognition of epithelial cells; (ii) access to epithelial cells; (iii) adhesion to epithelial cells; (iv) formation of permeation route and (v) migration to a

Abbreviation: TFP, type IV pili

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basolateral site. We have recently demonstrated that *P. aeruginosa* can penetrate the mucin layer using flagellar motility and mucin degradation [20]. We have also recently demonstrated that the type III effector molecule ExoS facilitates *P. aeruginosa* penetration through the epithelial barrier by impairing the defense function of tight junction proteins, including occludin, against bacterial penetration [17]. However, it remains unclear how *P. aeruginosa*, which has penetrated the mucus layer, then adheres to and injects the ExoS effectors into intestinal epithelial cells.

P. aeruginosa has two major structures that contribute to motility and adhesion: the flagella [21,22] and type IV pili (TFP) [23–25]. *P. aeruginosa* exhibits twitching motility, a form of translocation on a solid surface dependent on TFP [23]. This twitching motility derives from retraction of the filament, which mainly consists of the major pilus subunit protein PilA, or pilin [23,26,27]. PilT and PilU are PilB-like ATPases required for pilus retraction [28,29]. Deletion of the *pilA* gene reduces the association of *P. aeruginosa* with, and cytotoxicity to, human respiratory epithelial cells [22,30,31] and rabbit corneal epithelial cells [32]. Furthermore, the C-terminal region of pilin binds to Galβ1-4GlcNAcα of asialo-GM1 expressed by human respiratory epithelial cells [24,25,30,33]. However, the role of TFP in the penetration of *P. aeruginosa* through the intestinal epithelial cell barrier is currently unclear. Therefore, in the present study we examined whether *P. aeruginosa* penetrates the epithelial cell monolayer through the function of TFP.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The *P. aeruginosa* wild-type strain PAO1 [34], and mutant strains Δ*exoS* [17], Δ*fliC* [20], Δ*pilA* [35], and Δ*pilA*/*pilA* [35] are our laboratory stock strains. The *pilA* and *exoS* double mutant strain (Δ*pilA*Δ*exoS*) was constructed by insertion of Δ*pilA*-pEX18Tc into the Δ*exoS* strain [35]. A constructed pExoS-bla plasmid was modified from that reported previously [36]. Briefly, the promoter region and the GAP-encoding region of the *exoS* gene were PCR-amplified with primers *Bam*HI-*exoS* (5′-GCGCGGATCCACCTG CAGGCTGAGTACGCTCTCCTCG-3′) and *exoS*-Fu (5′-TTTCTGGG TGTCTAGACACTTCGGCGTCACTGTGGATGCCACC-3′). The *bla* gene was amplified from pGEX6P-1 (GE Healthcare, Waukesha, WI, USA) with primers *bla*-Fu (5′-CGCCGAAGTGTCTAGACACCCAGAA ACGCTGGTGAAAGTAAAAG-3′) and *Eco*R1-bla (5′-ATATGAATTCT TACCAATGCTTAATCAGTGAGGCACC-3′). These two flanking sequences were joined by fusion PCR using primers *Bam*HI-*exoS* and *Eco*R1-*bla* containing the engineered restriction sites *Bam*HI and *Eco*RI. To obtain the ExoS-Bla fusion protein expression plasmid, the *Bam*HI-*exoS*-*bla*-*Eco*RI fragment was ligated into the *Bam*HI-*Eco*RI fragment of pME6032 [37].

2.2. Antibodies

Anti-PilA polyclonal antibody was obtained from Scrum (Scrum, Tokyo, Japan) [35]. Anti-occludin antibody was obtained from Invitrogen (Life Technologies, Carlsbad, CA, USA). Anti-β-actin antibody was obtained from Chemicon (Merck Millipore, Billerica, MA, USA).

2.3. Bacterial penetration assay

We carried out a Caco-2 cell monolayer penetration assay as previously described [17]. Penetration by each strain was analyzed in triplicate and the experiment was replicated in triplicate.

2.4. Bacterial association assay

The assay was performed as reported previously [20]. Cellular association for each strain was analyzed in triplicate and the experiment was carried out in triplicate.

2.5. Expression of occludin protein in Caco-2 cell monolayers

The assay was performed as reported previously [17]. The presence of occludin in the samples was detected by Western blot analysis using anti-occludin (Life Technologies) and goat anti-rabbit IgG-peroxidase conjugated antibodies (Sigma, St. Louis, MO, USA). The bound antibodies were then visualized with the ECL prime Western blotting detection system (GE Healthcare).

2.6. Mucin penetration assay

We carried out an artificial mucin penetration assay as described previously [20]. Penetration of each strain was analyzed in triplicate and the experiment was replicated in triplicate.

2.7. CCF2-AM loading and β-lactamase detection

The assay was modified from a previously reported method [36]. Briefly, HeLa cells were seeded at 1×10^4 cells per well in a 96-well plate and incubated at 37 °C in 5% CO_2 for 2 days. Bacteria were inoculated at 1×10^6 CFUs per well. Following bacterial infection for 4 h, cells were washed six times with DPBS and incubated with freshly prepared CCF2-AM solution (1 μM final concentration; Invitrogen, Life Technologies) for 1 h in the dark at room temperature. Green fluorescence of the uncleaved substrate with an excitation wavelength of 410 nm and emission detection at 530 nm and blue fluorescence of the cleaved substrate with an excitation wavelength of 410 nm and emission detection at 450 nm were measured using a Varioskan™ microtiter plate reader (Thermo Scientific, Waltham, MA, USA). Cells were also observed using an IX-71 fluorescent microscope (Olympus, Tokyo, Japan). The data presented are the means ± standard deviations of three independent experiments.

2.8. Silkworm bacterial infection model

The assay was performed as reported previously [17]. In the oral infection model, 10 silkworms (*Bombyx mori*, Hu-Yo × Tukuba-Ne) at the fifth-instar stage were fed an antibiotic-free artificial food, Silkmate (an approximately 1.0 cm^3 block; Katakura Industries, Japan), to which 200 μl of a bacterial culture (10^{10} CFU/ml) grown overnight had been added. As a negative control, 200 μl of saline was added. The silkworms were maintained without food, and survival was monitored for 5 days. After infection for 6 h, the hemolymph of ten silkworms fed with *P. aeruginosa* was collected and analyzed for bacterial infection. Appropriate dilutions were spread onto LB agar plates, incubated at 37 °C overnight, and CFUs were counted to quantify bacteria. Invasion of each strain was analyzed in triplicate and the experiment was replicated in triplicate. Additionally wild-type and mutant strains of bacteria (10^3–10^7 CFU per silkworm) were injected into the hemolymph of 10 silkworms at the fifth-instar stage and viability monitored as above.

2.9. Statistical analyses

Results are presented as the mean ± standard deviation (S.D.). The data were subjected to a two-tailed *t*-test or one-way ANOVA using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). A *P*-value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. TFP are required for the penetration of P. aeruginosa through Caco-2 cell monolayers

Investigating penetration through intestinal epithelial cell monolayers using a Δ*pilA* and PAO1 strains demonstrated that following 3 h of infection bacteria of the Δ*pilA* strain could not penetrate Caco-2 cell monolayers while PAO1 strain could (Fig. 1A). The penetration through Caco-2 cell monolayers by the Δ*pilA* strain was rescued by introducing plasmids carrying the *pilA* gene (Fig. 1A). Type IV pili are required for the association of *P. aeruginosa* with human respiratory epithelial cells [22,30] and rabbit corneal epithelial cells [32]. We performed association assays with Caco-2 cells using the Δ*pilA* strain. There was no significant difference in the numbers of bacteria of either PAO1 or the Δ*pilA* strains, associated with Caco-2 cells (Fig. 1B). Furthermore, anti-PilA antibodies did not affect the number of bacteria associated with Caco-2 cells (Fig. 1C), although they decreased penetration through Caco-2 cell monolayers (Fig. 1D).

3.2. Disruption of Caco-2 cell junctions is necessary for TFP-dependent epithelial monolayer penetration

To penetrate the epithelial cell monolayer, bacteria must cross two major barriers; the mucous and epithelial cell layers [10–12]. To further examine the contribution of TFP in *P. aeruginosa* to penetration through Caco-2 cell monolayers, we performed an artificial mucin layer penetration assay using the pilus mutant strain. A decrease in penetration of the flagellar filament mutant, Δ*fliC*, was consistent with previous findings (Fig. 1E) [20]. However, there was no significant difference between PAO1 and the Δ*pilA* strains, which penetrated through an artificial mucin layer (Fig. 1E). Next, to determine whether *P. aeruginosa* can disrupt Caco-2 cell tight junctions through the function of TFP, we measured TER following infection with PAO1 and the Δ*pilA* strains. There was a steep drop in the TER of monolayers infected with PAO1 and the Δ*pilA*/*pilA* strains (Fig. 1F). There was no significant change in the TER following infection with the Δ*pilA* strain (Fig. 1F). TER is a sensitive measure of tight junctional barrier function and reflects the condition of tight junction formation. Western blot analysis revealed that infection of Caco-2 cells with PAO1 or the Δ*pilA*/*pilA* strain reduced the levels of expression of occludin protein at 4 h by 99% or 60%, respectively, and at 8 h by 99% or 98%, respectively, relative to that observed following infection with the Δ*pilA* strain (Fig. 1G). Overall, we observed a good correlation between changes in TER (Fig. 1F) and the degradation of occludin protein (Fig. 1G).

3.3. ExoS and TFP expression by the same bacterial cell is required for penetration of Caco-2 monolayers by P. aeruginosa

P. aeruginosa can disrupt Caco-2 cell tight junctions using TFP (Fig. 1A–G). A type III effector molecule, ExoS, facilitates *P. aeruginosa* penetration through the epithelial barrier by impairing the defense function of tight junctions [17,38]. To examine whether ExoS and TFP in the same bacterial cell are necessary for disruption of Caco-2 cell barriers, we measured the TER of Caco-2 cell monolayers following infection with a mixture of the Δ*pilA* and Δ*exoS* strains. There was no significant difference in TER changes among the mixture, each single mutant, and double mutant strains individually (Fig. 2A). Furthermore, the number of bacteria that penetrated through Caco-2 cell monolayers was positively correlated with the changes in TER (Fig. 2B). Our results showed that only *P. aeruginosa* expressing both ExoS and TFP in the same cell can disrupt epithelial tight junctions.

3.4. TFP are necessary for the injection of ExoS into host cells

To determine whether injection of ExoS by *P. aeruginosa* into epithelial cells is impacted by TFP-dependent cell penetration, translocation of ExoS effectors was detected directly within living host cells using the fluorescent β-lactamase substrate CCF2/AM. Green fluorescence was not observed in Caco-2 cells after treatment with CCF2/AM (data not shown), therefore we determined β-lactamase activity using HeLa cells, as reported previously [36]. HeLa cells were infected with *P. aeruginosa* strains containing a vector expressing an *exoS*/*blaM* (β-lactamase) fusion gene. CCF2 cleavage mediated by the translocated fusion protein induces the disruption of fluorescence resonance energy transfer and provokes a shift from green to blue fluorescence. Notably, deficiency of TFP reduced both the number of cells positive for the blue fluorescence derived from the degradation products of CCF2 (Fig. 3A), and also the ratio of blue to green fluorescence intensity by approximately 50% (Fig. 3B).

3.5. TFP are required for the virulence of P. aeruginosa after oral infection in silkworms

We performed an in vivo epithelial penetration assay using a silkworm bacterial infection model to determine whether the *pilA* and *exoS* genes are necessary for *P. aeruginosa* virulence after oral infection [17]. The virulence of the Δ*pilA*, Δ*exoS*, and Δ*exoS*Δ*pilA* strains after oral infection was significantly attenuated compared with the wild-type strain (Fig. 4A). We next directly evaluated the ability of PAO1, the Δ*pilA*, Δ*exoS*, and Δ*exoS*Δ*pilA* strains to penetrate the midgut barrier after oral administration. As shown in Fig. 4B, the numbers of bacteria of the Δ*pilA* and Δ*exoS* strains identified in the hemolymph at 6 h after oral infection were significantly lower than that of PAO1 strain. Additionally, the virulence after oral infection was similar between single and double mutant strains (Fig. 4A and B). Moreover, silkworm viability following the injection of bacteria into the hemolymph was similar among all strains at all inoculum doses tested (10^3–10^7 CFU/silkworm) (Fig. 4C and data not shown).

4. Discussion

Our data showed that TFP are required for efficient disruption of tight junctions after adhesion to epithelial cells by *P. aeruginosa* (Fig. 1A–G). Expression of both the type III effector ExoS and TFP in the same cell is required for the disruption of the tight junctions (Fig. 2A and B). Furthermore, ExoS injection by *P. aeruginosa* into epithelial cells is impacted by TFP expression, allowing penetration through the epithelial barrier (Fig. 3A and B). These in vitro findings were supported by results from an in vivo silkworm bacterial infection model (Fig. 4A–C). Collectively, these results suggest that TFP of *P. aeruginosa* are required for injection of the type III effector ExoS into epithelial cells and the penetration of *P. aeruginosa* through the epithelial barrier.

Deficiency of TFP reduced the amount of ExoS injected into epithelial cells (Fig. 3A and B). PilT is a hexameric ATPase required for bacterial type IV pilus retraction and surface twitching motility [23,26–29]. The twitching motility of *P. aeruginosa* is important for both *P. aeruginosa* cytotoxicity against epithelial cells and for virulence in vivo [23,39]. Our data showed that the deletion of TFP did not affect penetration through the artificial mucin layer and association with the epithelial cell surface (Fig. 1B and E). Based on our data and these findings, we hypothesize that *P. aeruginosa* injects

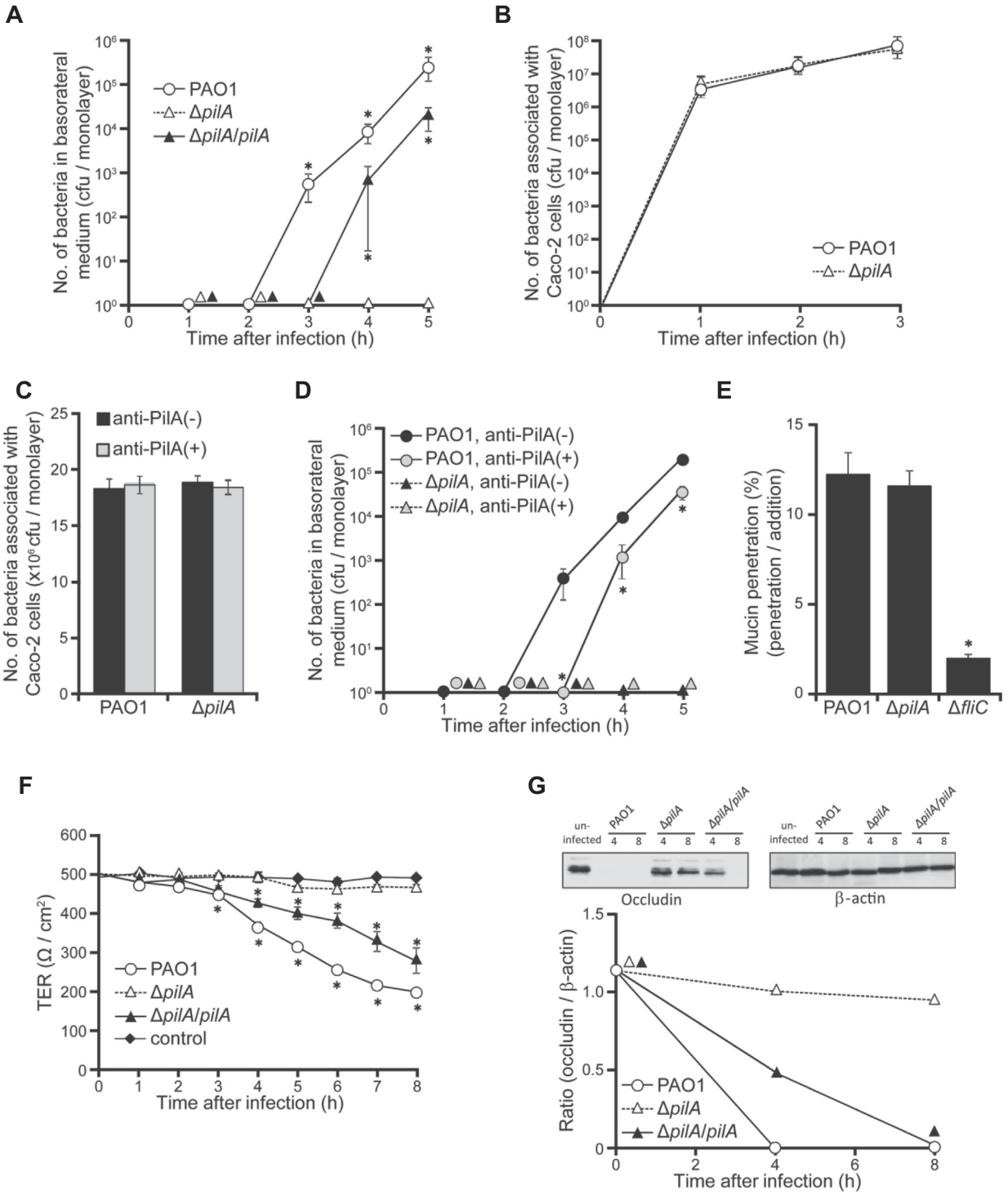

Fig. 1. Penetration of *P. aeruginosa* through epithelial cell monolayers in vitro. (A) Penetration of Caco-2 cell monolayers by wild-type *P. aeruginosa* (PAO1) and pilus mutant strains. Caco-2 cells were infected, and the number of bacteria in the Transwell basolateral medium counted. The assay was performed in triplicate, and the results are expressed as means ± S.D. $^{*}P < 0.05$ compared with TFP mutant strain (Δ*pilA*). (B) Association of wild-type and pilus mutant strains with Caco-2 cells. Bacteria were inoculated onto the apical surface of Caco-2 cells. The number of associated bacteria was evaluated by lysis of Caco-2 cells with Triton X-100 after washing of the Transwell with DPBS. The assay was performed in triplicate, and the results are expressed as means ± S.D. (C) *P. aeruginosa* strains were pretreated for 30 min with an anti-PilA antibody (1.6×10^5 CFUs/40 μg of antibody) then inoculated onto the apical surface of the Caco-2 cells. The number of associated bacteria was evaluated by lysis of Caco-2 cells with Triton X-100 after washing of the Transwell with DPBS. The assay was performed in triplicate, and the results are expressed as means ± S.D. (D) *P. aeruginosa* strains were pretreated for 30 min with an anti-PilA antibody (1.6×10^5 CFUs/40 μg of antibody) then inoculated onto the apical surface of Caco-2 cells in Transwells, and the number of bacteria in the basolateral medium counted. The assay was performed in triplicate, and the results are expressed as means ± S.D. $^{*}P < 0.05$ compared with the wild-type strain (PAO1) without anti-PilA antibody treatment. (E) Penetration of the mucin layer by the wild-type and flagellar and pilus mutant strains. Bacteria were loaded on top of 2% bovine submaxillary mucin, and the number of bacteria in the bottom medium counted at 1 h. The assay was performed in triplicate, and the results are expressed as means ± S.D. $^{*}P < 0.05$ compared with the wild-type strain (PAO1). (F) Changes in the TERs of Caco-2 cell monolayers. In the control, LB broth was added instead of bacteria. The TER was measured at the indicated times in triplicate and is expressed as the average ± S.D. of the resistance multiplied by the area. $^{*}P < 0.05$ compared with TFP mutant strain (Δ*pilA*). (G) Expression of occludin protein in Caco-2 cell monolayers infected with wild-type *P. aeruginosa* and pilus mutant strains. Caco-2 monolayers were infected and incubated. The cell lysates were analyzed by Western blotting using anti-occludin and anti-β-actin antibodies.

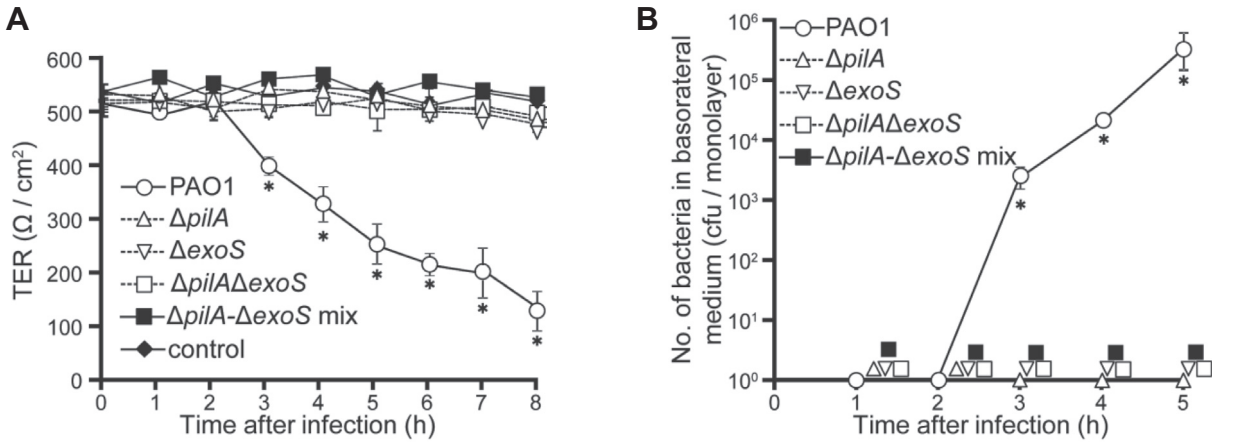

Fig. 2. Penetration of *P. aeruginosa* through epithelial cell monolayers using ExoS and TFP. (A) Changes in the TERs of Caco-2 cell monolayers induced by wild-type *P. aeruginosa*, the *pilA* gene mutant, the *exoS* gene mutant, the *pilA* and *exoS* genes double mutant, and a mixture of the *pilA* gene mutant and the *exoS* gene mutant strains. In the control, LB broth was added instead of bacteria. The TER was measured at the indicated times in triplicate and is expressed as the average ± S.D. of the resistance multiplied by the area. $^{*}P < 0.05$ compared with TFP mutant strain (Δ*pilA*). (B) Penetration of Caco-2 cell monolayers by wild-type *P. aeruginosa*, the *pilA* gene mutant, the *exoS* gene mutant, the *pilA* and *exoS* genes double mutant, and mixture of the *pilA* gene mutant and the *exoS* gene mutant strains. Caco-2 cells were infected and the number of bacteria in the basolateral medium counted. The assay was performed in triplicate, and the results are expressed as means ± S.D. $^{*}P < 0.05$ compared with TFP mutant strain (Δ*pilA*).

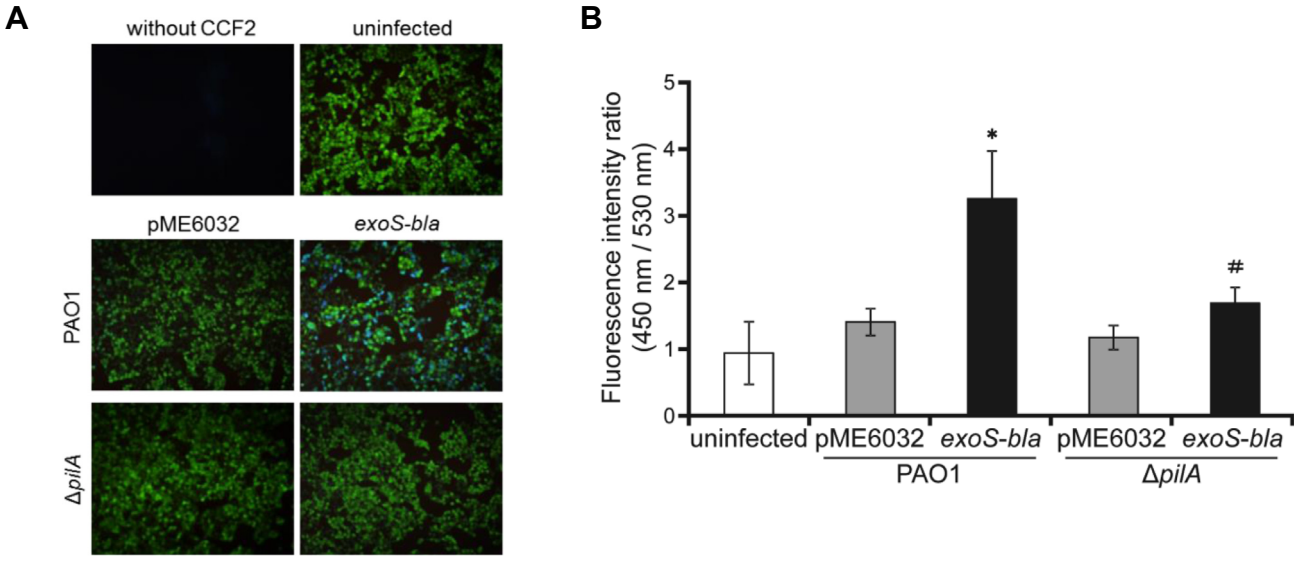

Fig. 3. ExoS injection by *P. aeruginosa* into epithelial cells is impacted by TFP dependent cell penetration. (A) HeLa cells were infected for 4 h with the wild-type, wild-type expressing ExoS-Bla, the pilus mutant, and the pilus mutant expressing ExoS-Bla. After incubation with β-lactamase substrate CCF2-AM, cells were observed by fluorescence microscopy using a 20× objective. (B) HeLa cells were infected for 4 h with the wild-type, wild-type expressing ExoS-Bla, the pilus mutant, and the pilus mutant expressing ExoS-Bla. After incubation with the β-lactamase substrate CCF2-AM, blue (450 nm) and green (530 nm) fluorescence intensities of the cells were measured with a fluorometric plate reader. The extent of ExoS-bla injection into cells is shown as the blue (450 nm)/green (530 nm) fluorescence ratios. The assay was performed in triplicate, and the results are expressed as means ± S.D. $^{*}P < 0.05$ compared with PAO1 harboring the pME6032 plasmid. $^{\#}P < 0.05$ compared with PAO1 expressing ExoS-Bla.

ExoS into epithelial cells via the twitching motility on the epithelial cell surface. This hypothesis will be addressed in future studies.

P. aeruginosa has two major structures that contribute to motility: the flagella [21,22] and TFP [23–25]. Moreover, other motility mechanisms have been suggested in *P. aeruginosa* [40]. Our recent report suggested that *P. aeruginosa* uses some form of motility other than the flagella to penetrate through the mucin layer, because we observed mucin layer penetration even in the absence of an intact flagellum [20]. In this study, deficiency of TFP did not affect penetration through the artificial mucin layer and association with the epithelial cell surface (Fig. 1B–E). Taken together, TFP are not required for penetration of mucin layers. Therefore, further studies are necessary to better understand the motility used for penetration of mucin layers.

The flagella and TFP are adhesins for host cells [22]. Other adhesins have been identified, including the cup fimbrial adhesins [41] and lectins PA-IL (LecA) and PA-IIL (LecB) [42]. In this study, we observed association of bacteria with Caco-2 cells even in the absence of intact pili. Taken together, *P. aeruginosa* can bind to Caco-2 cells using other adhesion systems. However, further studies are necessary to understand the role of *P. aeruginosa* adhesins in adherence to Caco-2 cells.

P. aeruginosa can adhere to human respiratory [22] and rabbit corneal [32] epithelial cells using TFP. However, we observed that there was no difference in association with Caco-2 human intestinal cells between bacteria of the wild-type and the pilus mutant strains (Fig. 1B and C). This may be cell specific. For example, if asialo-GM1, to which the pilin binds [24,25,33], varies in

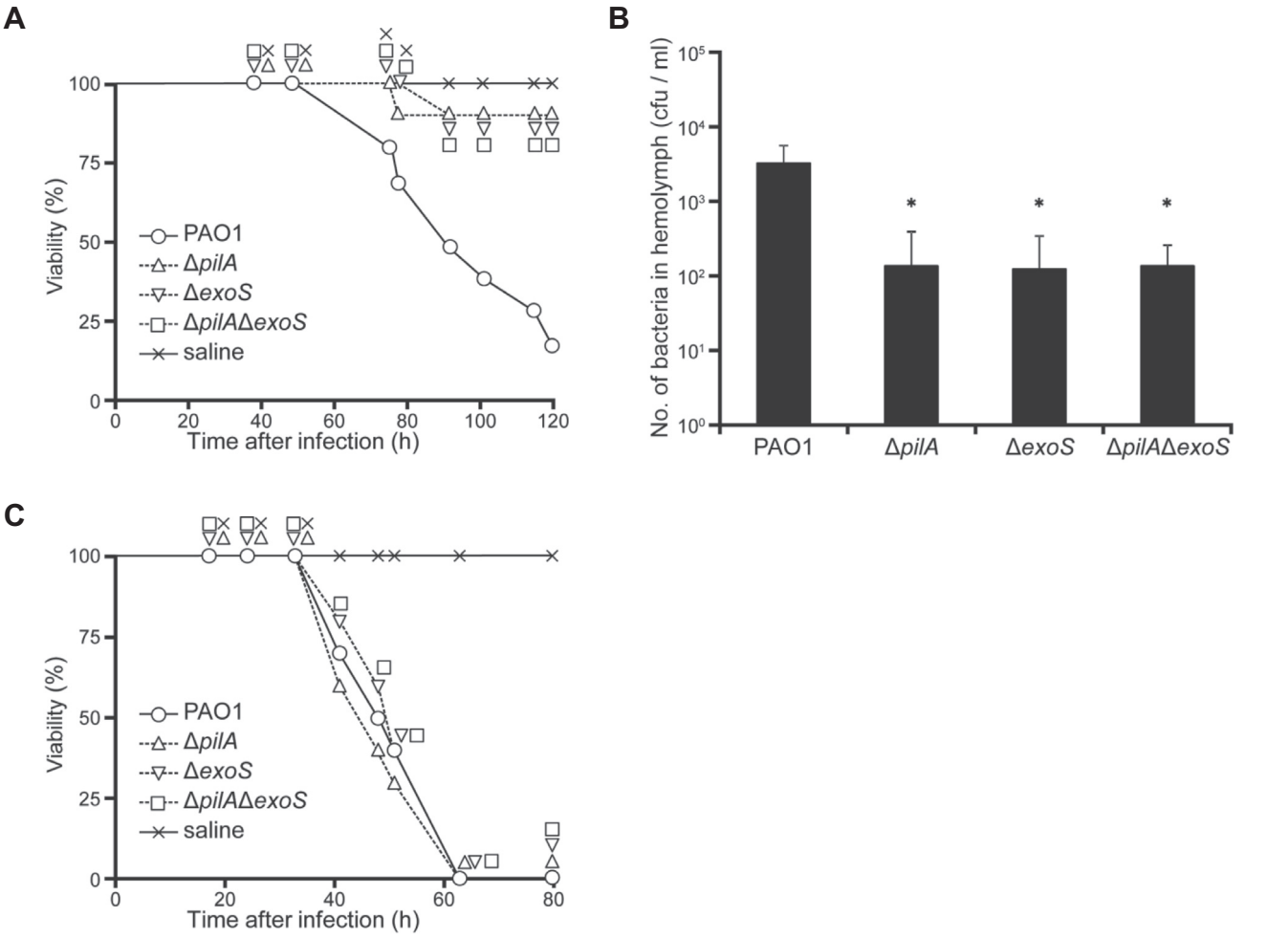

Fig. 4. Pili are essential for *P. aeruginosa* virulence in silkworms because of its ability to mediate penetration through the midgut epithelial barrier. (A) Comparison of the virulence of wild-type *P. aeruginosa*, *pilA* gene mutant, *exoS* gene mutant, and the *pilA* and *exoS* genes double mutant strains following oral administration to 10 silkworms. Saline was used as a control. (B) Numbers of bacteria in the hemolymph at 6 h after oral administration of wild-type *P. aeruginosa*, *pilA* gene mutant, *exoS* gene mutant, and the *pilA* and *exoS* genes double mutant strains to 10 silkworms. The assay was performed in duplicate, and the results are expressed as means ± S.D. $^{*}P < 0.05$ compared with the wild-type strain (PAO1). (C) Comparison of the virulence of wild-type *P. aeruginosa*, *pilA* gene mutant, *exoS* gene mutant, and the *pilA* and *exoS* double mutant strains following injection into the hemolymph (10^3 CFU per silkworm) of 10 silkworms. Saline was used as a control.

expression or localization pattern between Caco-2 cells and other cells. However, the reasons for the inability of *P. aeruginosa* to associate with Caco-2 cells via TFP remain unclear.

Fig. 4A and B showed that TFP are required for *P. aeruginosa* translocation from the gut to the hemolymph of silkworms. Fig. 4C showed that silkworm viability following the injection of bacteria into the hemolymph was similar between PAO1 and the Δ*pilA* strains. Furthermore, there was a positive correlation between the number of bacteria in the hemolymph and the mortality rate of silkworms after direct injection of *P. aeruginosa* into hemolymph [43–45]. Therefore, we conclude that the mortality of silkworms following oral infection by the wild-type strain is at least partially dependent upon the ability of the bacteria to penetrate efficiently through the gut epithelial barrier in a TFP-dependent manner. Additionally, because there was no significant difference between groups after infection with the Δ*pilA*, Δ*exoS*, and Δ*pilAexoS* strains (Fig. 4A–C), we hypothesize that both TFP and ExoS are necessary for the establishment of gut-derived sepsis after oral infection by the same pathway.

In summary, our study using in vitro cultured epithelial cells and an in vivo silkworm bacterial infection model, showed that the *P. aeruginosa* PAO1 strain requires TFP for the injection of the type III effector molecule ExoS into epithelial cells by the type III secretion system, thereby enabling penetration of intestinal epithelial barriers. Further studies will be required to clarify the contribution of TFP to *P. aeruginosa* penetration through epithelial tissues, although these findings may lead to new therapeutic strategies for *P. aeruginosa* gut-derived sepsis.

Author contributions

NH and NG designed experiments; NH, HN, SK, SD, TN, AF, and MS performed experimets; NH analyzed data; NH wrote the manuscript; NH and NG made manuscript revisions; NH and NG conceived and supervised the study.

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