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Type IV Pilus of *Pseudomonas aeruginosa* Confers Resistance to Antimicrobial Activities of the Pulmonary Surfactant Protein-A

Rommel Max Tan Zhizhou Kuang Yonghua Hao Gee W. Lau

Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, Ill., USA

Key Words
Surfactant protein-A · *Pseudomonas aeruginosa* · Type IV pilus · Opsonization · Membrane permeabilization · Adhesins

Abstract
Pseudomonas aeruginosa (PA) is a Gram-negative bacterial pathogen commonly associated with chronic lung infections. Previously, we have identified several *PA* virulence factors that are important for resistance to the surfactant protein-A (SP-A), a pulmonary innate immunity protein that mediates bacterial opsonization and membrane permeabilization. In this study, we demonstrate that the type IV pilus (Tfp) is important in the resistance of *PA* to the antibacterial effects of SP-A. The Tfp-deficient mutant *ΔpilA* is severely attenuated in an acute pneumonia model of infection in the lungs of wild-type mice, but is virulent in the lungs of SP-A$^{-/-}$ mice. The *ΔpilA* bacteria are more susceptible to SP-A-mediated aggregation and opsonization. In addition, the integrity of the outer membranes of *ΔpilA* bacteria is compromised, rendering them more susceptible to SP-A-mediated membrane permeabilization. By comparing Tfp extension and retraction mutants, we demonstrate that the increased susceptibility of *ΔpilA* to SP-A-mediated opsonization requires the total absence of Tfp from *PA* cells. Finally, we provide evidence of increased expression of nonpilus adhesin OprH that may serve as an SP-A ligand, resulting in increased phagocytosis and preferential pulmonary clearance of *ΔpilA*.

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Introduction

Pseudomonas aeruginosa (PA) is an opportunistic Gram-negative bacterial pathogen commonly associated with acute or chronic infection of mechanically damaged (ventilator-associated pneumonia), immunocompromised (HIV, malignancies and immunosuppressive drugs) and mechanically obstructed (cystic fibrosis and chronic obstructive pulmonary disease) lungs [1]. In addition to lung infections, *PA* is also prevalent in contact-lens-associated keratitis and is a major cause of burn infections [2, 3]. The prevalence of *PA* in many infections can be attributed to its ability to express a wide array of virulence factors and form biofilms [4] as well as its intrinsically high levels of resistance to many antibiotics [5].

Among the virulence factors expressed by *PA* is the unipolarly localized surface appendage, type IV pilus (Tfp) [4]. Tfp deficiency attenuates the ability of *PA* to

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Dr. Gee W. Lau
Department of Pathobiology, University of Illinois at Urbana-Champaign
2001 South Lincoln Ave
Urbana, IL 61802 (USA)
E-Mail geelau@illinois.edu

induce epithelial cytotoxicity, pneumonia, septicemia and mortality in mice [6, 7]. Tfp is important for twitching motility, epithelial adhesion, substratum attachment and biofilm formation as well as phage attachment and uptake [4, 8]. Tfp is made up of monomers of pilin, encoded by the *pilA* gene [8, 9]. The assembly and disassembly of Tfp are powered by the ATPases PilB and PilT/U, respectively, producing twitching motility [8, 10]. These extension and retraction proteins are regulated by the Chp chemosensory system [10]. The asialoGM1 [4, 8, 9, 11] and N-glycan chains [12, 13] located on the apical surface of the host epithelium are the main receptors for Tfp.

The surfactant of the lung contains phospholipids and four major surfactant proteins (SPs): SP-A, B, C and D [14, 15]. The naturally occurring octadecameric SP-A is the most abundant [14, 15]. Each monomer contains an N-terminal, triple-helical collagen region that binds to eukaryotic receptor (SP-A receptor 210), and a C-terminal carbohydrate-recognition domain (CRD) that binds to microbial carbohydrates [14–16]. Binding of the CRD to microbial carbohydrates aggregates microbes, enhancing their phagocytosis [14, 17–21]. In addition to opsonic phagocytosis, SP-A enhances phagocytosis directly [15, 22]. SP-A$^{-/-}$ mice are more susceptible to lung infection, with decreased bacterial clearance and reduced macrophage phagocytosis [15, 23, 24]. Most recently, we and others have shown that SP-A directly permeabilizes microbial membranes [17–21].

Previously, our laboratory has shown that several virulence factors of *PA*, including lipopolysaccharide (LPS), phosphoenolpyruvate transferase, isochorismate synthase, flagellum and elastase B confer resistance to SP-A-mediated antimicrobial effects [18–21]. In this paper, we show that Tfp of *PA* also plays a major role in resistance to SP-A-mediated phagocytosis and membrane permeabilization.

Materials and Methods

Bacterial Strains and Growth Conditions

The wild-type *PA* strain PAO1 and its isogenic derivatives *ΔpilA*, the genetically-complemented strain *ΔpilA*-comp, *ΔchpA*, *ΔpilG*, *ΔpilB*, *ΔpilH*, *ΔpilT* and *ΔpilU* were generously provided by Prof. Joanne Engel (University of California, San Francisco, Calif., USA) [10]. The *rpoN::ISphoA/hah* mutant was purchased from the University of Washington Genome Sciences [25] (table 1). *PA* strains were grown in Luria-Bertani (LB) broth and stored at –80 °C in 30% glycerol. Before each experiment, bacteria were streaked from frozen stock onto LB agar for 18 h at 37 °C. One colony from this streak was then cultured in 5 ml LB broth to the stationary phase (OD_{600} approx. 3.0).

Mouse Clearance Assay

Wild-type C3H/HeN (SP-A$^{+/+}$) mice were purchased from Harlan Laboratory (South Easton, Mass., USA). Isogenic SP-A$^{-/-}$ mice were gifts from Dr. Francis McCormack (University of Cincinnati College of Medicine, Cincinnati, Ohio, USA). Mouse experiments complied with the guidelines and were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. SP-A$^{+/+}$ and SP-A$^{-/-}$ mice (n = 5) were given a single intranasal inoculation of 1×10^7 PAO1 or *ΔpilA* strain. After 18 h of infection, mouse lungs were harvested for histology or bacterial enumeration as we previously described [18–21].

In vivo Phagocytosis Assay

The phagocytosis rates between different *PA* strains were compared using the gentamicin exclusion assay as previously described [21]. Briefly, SP-A$^{+/+}$ and SP-A$^{-/-}$ mice (n = 3) were given a single intranasal inoculation of 1×10^7 PAO1 or *ΔpilA* cells. After 2 h, mouse lungs were lavaged to collect the alveolar macrophages and neutrophils. The macrophages were then incubated in PBS supplemented with 100 μg/ml gentamicin to kill the remaining extracellular bacteria. The macrophages were lysed with 1% Triton X-100 solution and serially diluted for the enumeration of internalized *PA*. The ratio of CFU counts between SP-A$^{+/+}$ and SP-A$^{-/-}$ mice was computed for the fold increase of phagocytosis mediated by SP-A.

Purification of Human SP-A

Discarded lung washings from anonymous alveolar proteinosis patients were generously provided by Dr. Francis McCormack. Human (h)SP-A was purified as previously described [26]. Briefly, raw lung washings, equilibrated with 1 mM $CaCl_2$, were passed through a Sepharose® 6B column laden with mannose. The captured SP-A was eluted using the elution buffer (2 mM EDTA and 5 mM Tris-HCl, pH 7.4). The eluted fractions were dialyzed using the dialysis buffer (150 mM NaCl and 5 mM Tris-HCl, pH 7.4) to remove EDTA. The purity of hSP-A preparations was confirmed by Coomassie blue stain.

Murine Macrophage Cell Line and in vitro Phagocytosis Assay

Murine RAW 264.7 macrophages (ATCC# TIB-71) were maintained in DMEM supplemented with 10% FBS at 5% CO_2 and 37 °C [21]. The phagocytosis rates between different *PA* strains were compared using the gentamicin exclusion assay. Briefly, 1×10^6 RAW 264.7 macrophages/ml were plated in 6-well cell culture plates overnight in a 37 °C incubator with 5% CO_2. *PA* strains were preincubated with 12.5, 25 or 50 μg/ml hSP-A in the presence of 2 mM $CaCl_2$ for 1, 6 or 12 h in a shaker (300 rpm) at 37 °C. The resulting mixture was then incubated with the RAW 264.7 cells at a ratio of bacteria/macrophages of 10:1 for 1.5 h. The macrophages were then washed and incubated in fresh DMEM supplemented with 100 μg/ml gentamicin to kill the remaining extracellular bacteria. The macrophages were lysed with 1% Triton X-100 solution, and serially diluted for enumeration. The ratio of CFU between treated and untreated bacteria was computed for the fold increase of phagocytosis mediated by hSP-A.

Aggregation Assay

PA strains were transformed with the plasmid pUCP19 harboring a green fluorescent protein (GFP) gene by electroporation as previously described [21]. Bacterial aggregation was performed using the stationary-phase *PA* incubated with 25 μg/ml hSP-A and

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
P. aeruginosa strains		
PAO1	wild-type	[10]
PAO1 *ΔpilA*	in-frame deletion of the *pilA* gene	[10]
ΔpilA-comp	genetically-complemented *ΔpilA* mutant	[10]
PAO1-GFP	PAO1 harboring pUCP19-*gfp*	this study
ΔpilA-GFP	*ΔpilA* harboring pUCP19-*gfp*	this study
ΔchpA	in-frame deletion of the *chpA* gene	[10]
ΔpilG	in-frame deletion of the *pilG* gene	[10]
ΔpilB	in-frame deletion of the *pilB* gene	[10]
ΔpilH	in-frame deletion of the *pilH* gene	[10]
$\Delta pilT^{\text{CTX-pilU}}$	in-frame deletion of the *pilT*; *pilU* and 1 kb upstream sequence at *attB* site	[10]
ΔpilU	in-frame deletion of the *pilU* gene	[10]
rpoN::ISphoA/hah	mutant harboring a *ISphoA/hah* transposon insertion into the *rpoN* gene	University of Washington Genome Sciences
Plasmids		
pUCP19-*gfp*	plasmid pUCP19 expressing a GFP	[21]

2 mM $CaCl_2$ for 1 h in a shaker (300 rpm) at 37°C. Bacterial clusters were enumerated from 10 independent fields under a fluorescence microscope.

Membrane Permeabilization Assay

The membrane permeability effects of hSP-A were measured using both the thiol-specific fluorophore ThioGlo® (Calbiochem, San Diego, Calif., USA) and enzyme-labeled fluorescence phosphatase substrate ELF-97® (Molecular Probes, Carlsbad, Calif., USA), as previously described [17, 20, 21]. Stationary-phase *PA* bacteria were washed and incubated with either 50 µg/ml hSP-A for 15 min at 37°C or with 50 µg/ml total protein of bronchoalveolar lavage fluids (BALF) from either SP-$A^{+/+}$ or SP-$A^{-/-}$ mice. For the ThioGlo assay, the bacteria/SP-A mixture was sedimented, and the supernatant was incubated with 10 µM ThioGlo. Fluorescence was measured at an excitation wavelength of 405 nm and an emission wavelength of 535 nm. For the ELF-97 assay, the bacteria/SP-A mixture was directly incubated with 100 µM ELF-97. Fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 535 nm every 3 min for a period of 90–120 min.

Bacterial Viability Assay

Stationary-phase *PA* bacteria were washed and incubated with either 50 µg/ml hSP-A or 50 µg/ml total protein of BALF from either SP-$A^{+/+}$ or SP-$A^{-/-}$ mice for 60 min or 6 h at 37°C. The bacteria were then stained with a combination of SYTO® 9 and propidium iodide from the commercial bacterial viability kit, LIVE/DEAD® BacLight (Molecular Probes). The numbers of dead and live bacteria were counted in 10 independent high-power fields (×400) using a fluorescent microscope.

Elastase Assay

Elastase production in the culture supernatant of stationary-phase *PA* was evaluated using the Sensolyte™ Red Protease assay kit (AnaSpec Inc., San Jose, Calif., USA). Fluorescence was measured at excitation 546 nm and emission 575 nm.

Pyocyanin Assay

Pyocyanin in the bacteria-free supernatant of stationary-phase *PA* cultured in LB broth or a low-phosphate medium (20 mM succinic acid, 40 mM NH_4Cl, 2 mM K_2SO_4, 0.014 g NAH_2PO_4, 1 M MOPS, 40 mM $MgCl_2$, 100 mM $CaSO_4$, 100 mM $ZnCl_2$, 30 mM $MnCl_2$ and 30 mM $Fe(NO_3)$) were measured at OD_{690} [20].

SDS Lysis Assay

To assess the membrane stability of *PA* strains, bacterial cell lysis was performed with 0.25% SDS as previously described [19]. Stationary-phase *PA* bacteria (OD_{600} 3.0) were washed and resuspended in PBS with 0.25% SDS. The OD_{600} was measured every 10 min for 1 h.

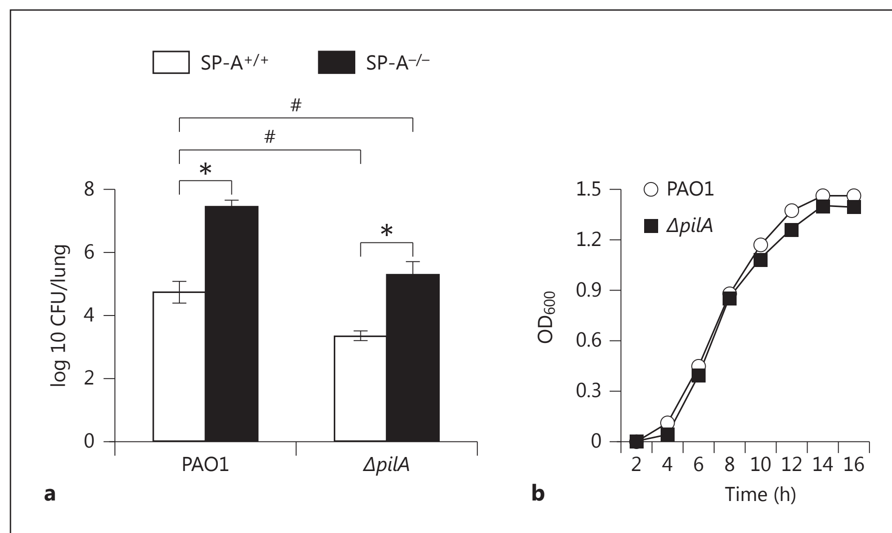
LPS Analysis

LPS was purified from stationary-phase *PA* strains as follows: the bacteria were sedimented and resuspended in 200 µl lysis buffer (2 g SDS, 4 ml 2-mercaptoethanol, 0.003 g bromophenol blue and 1 M Tris-Hcl, pH 6.8) and boiled for 10 min. Three microliters of 20 µg/µl proteinase K were added, and the mixture was incubated for 1 h at 60°C. The resulting mixture was separated in an SDS-PAGE gel. The resolved gel was incubated with a fixing solution (25% v/v isopropyl alcohol and 7% v/v acetic acid) overnight at 4°C, oxidized (0.7% periodic acid, 2.7% ethanol and 0.3% v/v acetic acid) for 5 min with gentle agitation, and washed with water 3 times for 30 min each. The gel was then placed in staining solution (4% v/v 1 M NaOH, 5% v/v NH_4OH and 2% $AgNO_3$) and shaken for 10 min, followed by four washes in water for 10 min each. Finally, the gel was developed (0.02% citric acid and 0.05% v/v formaldehyde) for 20 min. The development was stopped using a stop solution (0.8% v/v acetic acid).

Transmission Electron Microscopy

PA strains were grown to OD_{600} approximately 3.0 in LB broth at 37°C. Bacteria were washed with PBS, and subsequently fixed

Fig. 1. *ΔpilA* bacteria are more susceptible to clearance by SP-A. **a** Respiratory tract infections with wild-type PAO1 versus *ΔpilA* were performed by intranasal inoculation of anesthetized SP-$A^{+/+}$ or SP-$A^{-/-}$ mice. Mouse lungs were harvested 18 h after infection for bacterial enumeration. Data are the mean CFU ± SE (n = 5 per group). * $p < 0.05$ when comparing the bacterial loads between SP-$A^{+/+}$ and SP-$A^{-/-}$ infected by PAO1 or *ΔpilA*. # $p < 0.05$ when comparing the bacterial loads between PAO1 and *ΔpilA* infecting the SP-$A^{+/+}$ or SP-$A^{-/-}$ mice. **b** The growth kinetics of PAO1 and *ΔpilA* bacteria were determined by measuring OD_{600}. The experiments were performed independently in triplicate. The representative growth curve from 1 of 3 independent experiments is shown.



using the Karnovsky fixative. Transmission electron microscopy (TEM) was performed at the University of Illinois Material Research Laboratory.

Ligand Blot

Biotinylated SP-A was used to identify potential receptors in *PA* strains as previously described [16]. Briefly, stationary-phase bacteria were ruptured under 14,000 psi in a French press. Membranes were isolated in 100 mM sodium carbonate and centrifuged at 115,000 *g*. Isolated membranes were solubilized using 50 mM Tris pH 7.4 mixed with loading buffer, boiled for 5 min and then resolved using 15% SDS-PAGE gels. Gels were either stained using Coomassie blue or transferred to PVDF membranes. Membranes were blocked for 2 h, and subsequently incubated with 3 μg/ml biotinylated SP-A. After 3 washings, streptavidin-conjugated HRP was added. Signal was detected using commercially available Western blot stain and substrate. Ligand blot signal was analyzed with the Image J software. Corresponding bands in gels stained with Coomassie blue to the signal detected in the PVDF membrane were sent for analyses at the University of Illinois LC-MS/MS core facility.

Results

Tfp Is Important in Resistance to SP-A-Mediated Lung Clearance

To determine the contribution of Tfp-mediated resistance to SP-A, we compared the virulence of the wild-type *PA* PAO1 and the isogenic *ΔpilA* mutant in a mouse model of acute pneumonia. Eighteen hours after intranasal inoculation with PAO1 or *ΔpilA*, SP-$A^{+/+}$ mice showed signs of infection and respiratory distress but were not moribund. In contrast, PAO1-infected SP-$A^{-/-}$ mice were moribund and had to be euthanized (data not shown). The number of viable PAO1 or *ΔpilA* bacteria in SP-$A^{-/-}$ were 2.74 log and 1.97 log higher than in SP-$A^{+/+}$ mice, respectively (fig. 1a). The number of *ΔpilA* bacteria in SP-$A^{-/-}$ mice was statistically indistinguishable when compared to the number of PAO1 in the SP-$A^{+/+}$mice, suggesting that *ΔpilA* was more virulent during lung infection in the absence of SP-A. However, the viable counts of *ΔpilA* were 1.4 log lower than PAO1 in SP-$A^{+/+}$ mice and 2.16 log lower than PAO1 in SP-$A^{-/-}$ mice. Because the relative decrease of bacterial load between PAO1 and *ΔpilA* from SP-$A^{-/-}$ mice to SP-$A^{+/+}$ mice was only 0.77 log, this suggests that *ΔpilA* is in general less virulent. In addition, other innate immune factors of the lung within the alveolar space may compensate for the absence of SP-A. Thus, a straightforward link between Tfp and SP-A cannot be easily established (fig. 1a). The *ΔpilA* showed similar growth kinetics to that of PAO1, suggesting that the difference in mouse clearance was not due to the growth defects in the former (fig. 1b).

The aforementioned quantitative observations were supported by histopathology (fig. 2). In the absence of bacterial infection, histopathological features of SP-$A^{-/-}$ mouse lungs were indistinguishable when compared to the lungs of SP-$A^{+/+}$ mice [data not shown]. PAO1 caused moderate bronchopneumonia (fig. 2a) whereas the *ΔpilA* mutant only caused mild bronchopneumonia in the lungs of SP-$A^{+/+}$ mice (fig. 2b). In contrast, PAO1 caused severe bronchopneumonia in SP-$A^{-/-}$ mice, with large amounts of pulmonary infiltrates and consolidation (fig. 2c). Importantly, *ΔpilA* caused more severe pulmonary infiltrate in SP-$A^{-/-}$ mice (fig. 2d) than the SP-$A^{+/+}$ mice (fig. 2b). The severity of *ΔpilA*-mediated bronchopneumonia in SP-$A^{-/-}$ lungs (fig. 2d) was similar to that caused by PAO1

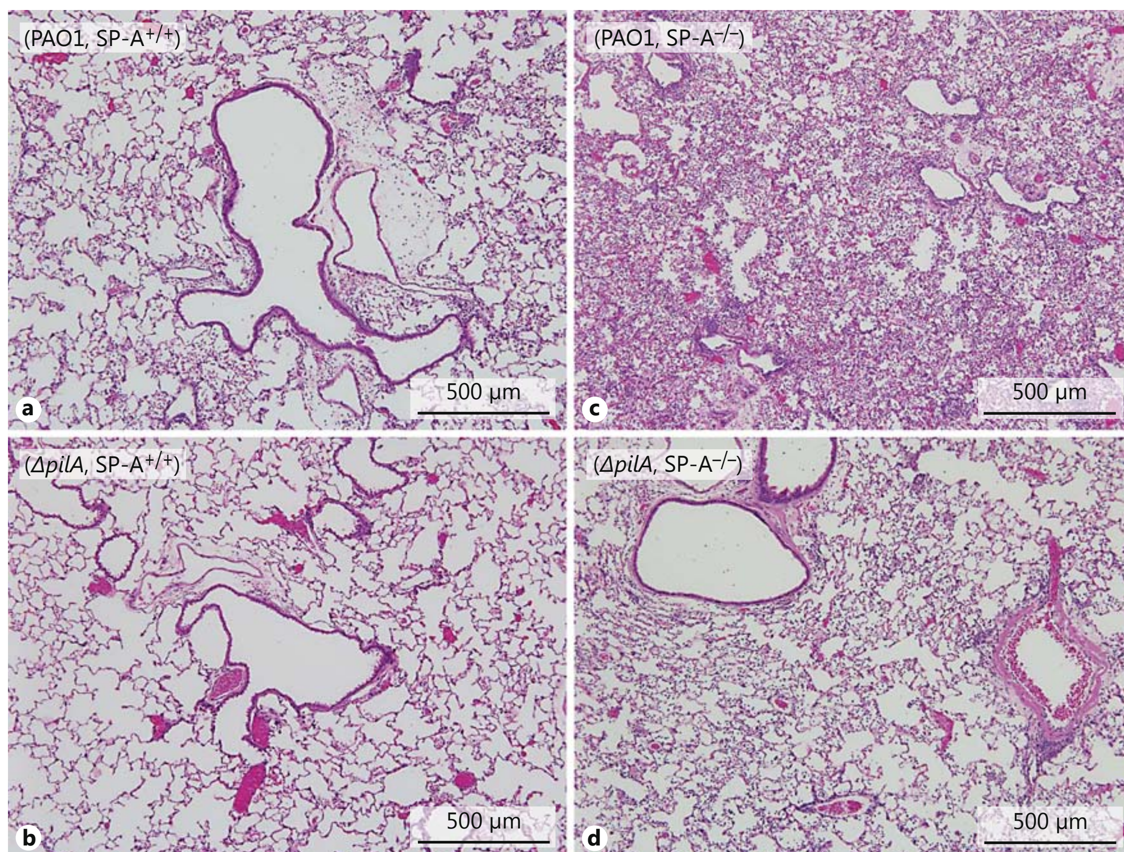


Fig. 2. Histopathology of *PA*-infected lungs. SP-$A^{+/+}$ and SP-$A^{–/–}$ mice were infected with PAO1 or *ΔpilA* as described in figure 1. Representative HE-stained lung sections from SP-$A^{+/+}$ and SP-$A^{–/–}$ mice (n = 5) 18 h after intranasal instillation of PAO1 (**a**, **c**) and *ΔpilA* (**b**, **d**) bacteria.

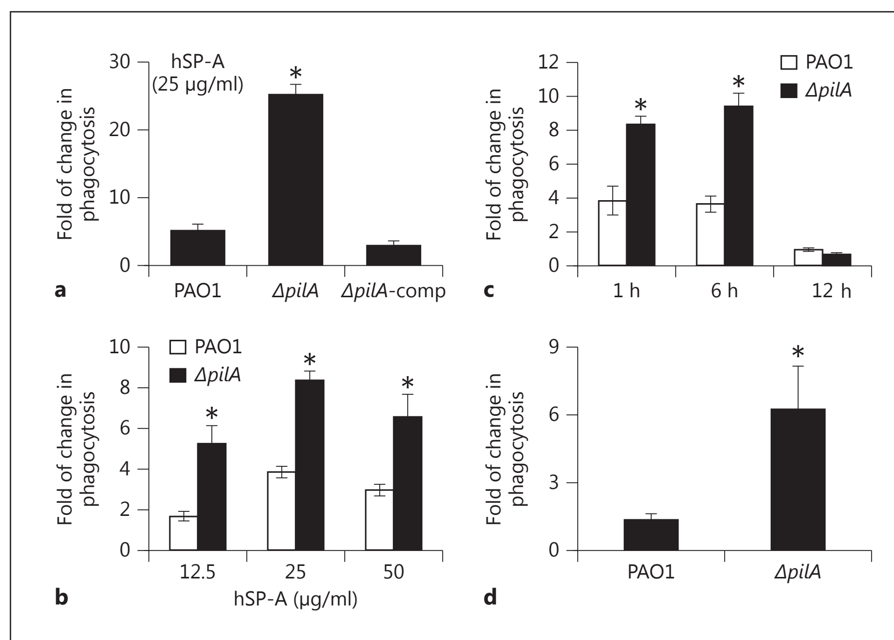


Fig. 3. The *ΔpilA* mutant is more susceptible to SP-A-mediated opsonization. **a–c** RAW 264.7 macrophages were infected with either PAO1 or *ΔpilA* in the presence or absence of hSP-A. The ratio of ingested bacteria was expressed as fold increase in phagocytosed bacteria due to the effect of hSP-A. **a** Phagocytosis of PAO1, *ΔpilA* and genetically complemented *ΔpilA*-comp in the presence of 25 µg/ml hSP-A. **b** Phagocytosis of PAO1 and *ΔpilA* in the presence of different concentrations of hSP-A. **c** Time-dependent phagocytosis of PAO1 versus *ΔpilA* in the presence of 25 µg/ml hSP-A. **d** In vivo phagocytosis of PAO1 versus *ΔpilA* in SP-$A^{+/+}$ and SP-$A^{–/–}$ mice (n = 3). All phagocytosis experiments were independently performed in triplicate. The mean ± standard deviation from 1 representative experiment is shown. * $p < 0.05$ when comparing the number of phagocytosed *ΔpilA* to PAO1.

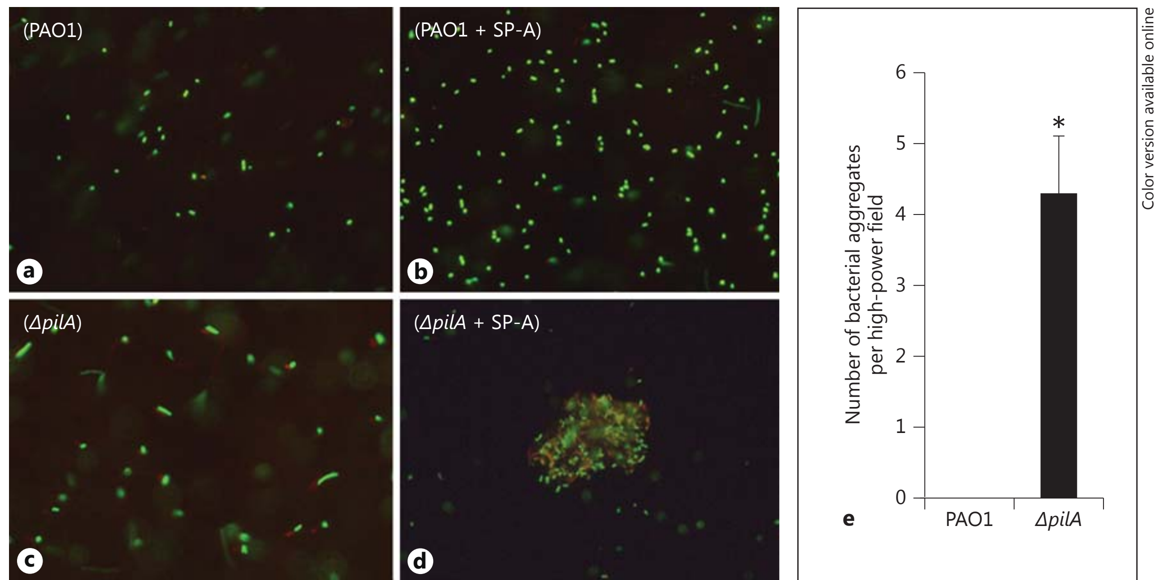


Fig. 4. *ΔpilA* are more susceptible to SP-A-mediated aggregation. GFP-expressing PAO1 and *ΔpilA* bacteria were incubated with 25 μg/ml hSP-A for 1 h and examined under a confocal fluorescence microscope. **a** PAO1 without hSP-A. **b** PAO1 with hSP-A. **c** *ΔpilA* without hSP-A. **d** *ΔpilA* with hSP-A. **e** The number of aggregates was averaged from 10 independent high-power fields. * $p < 0.05$ when comparing the number aggregates in *ΔpilA* to PAO1.

in SP-A$^{+/+}$ lungs (fig. 2a), suggesting that the virulence levels of *ΔpilA* in mouse lungs devoid of SP-A was similar to PAO1 in mouse lungs with sufficient SP-A. Collectively, these results indicate that Tfp plays a protective role against anti-*PA* activity mediated by SP-A.

Tfp Is Important for Resistance of SP-A-Mediated Phagocytosis

To decipher the mechanism of Tfp resistance in SP-A-mediated lung clearance, we examined whether *ΔpilA* bacteria were more susceptible to SP-A-mediated opsonization. In the presence of 25 μg/ml of SP-A, *ΔpilA* bacteria were phagocytized 5 times more efficiently than the wild-type PAO1 (fig. 3a). The genetically-complemented *ΔpilA-comp* bacteria were as resistant to SP-A-mediated opsonization as PAO1 (fig. 3a). These results were confirmed by studies using different concentrations of SP-A, which consistently showed that, in the presence of SP-A, *ΔpilA* was more susceptible to phagocytosis by macrophages than PAO1 (fig. 3b). We also examined the phagocytosis of *ΔpilA* in a time-dependent manner. Again, *ΔpilA* was more susceptible to SP-A-mediated opsonization at 1 and 6 h after exposure to RAW 264.7 macrophages (fig. 3c). However, prolonged exposure (12 h) abolished phagocytosis, most probably because both PAO1 and *ΔpilA* bacteria degraded SP-A by secreting exoproteases, as we have previously described [20, 21]. These observations were confirmed by an in vivo phagocytosis assay, which showed that *ΔpilA* was 4.5 times more susceptible to SP-A-mediated opsonization than PAO1 (fig. 3d). The in vivo phagocytosis assay measures the total phagocytosis activity involving both alveolar macrophages and infiltrating neutrophils responding to *PA* infection. Collectively, these results indicate that Tfp is important for resistance to SP-A-mediated opsonization.

Tfp Is Important for Resistance to SP-A-Mediated Aggregation

One of the mechanisms by which SP-A enhances microbial phagocytosis is by increasing their aggregation, allowing more efficient phagocytosis [22]. We compared the aggregation of wild-type PAO1 versus *ΔpilA* by SP-A in vitro. The *ΔpilA* mutant bacteria were aggregated by SP-A 4-fold higher than PAO1 per high-power field (fig. 4a–e). Overall, these results show that Tfp confers resistance to SP-A-mediated aggregation.

Tfp Is Important for Resistance to SP-A-Mediated Membrane Permeabilization

Apart from its ability to opsonize and facilitate the phagocytosis of microbes by macrophages, SP-A is also

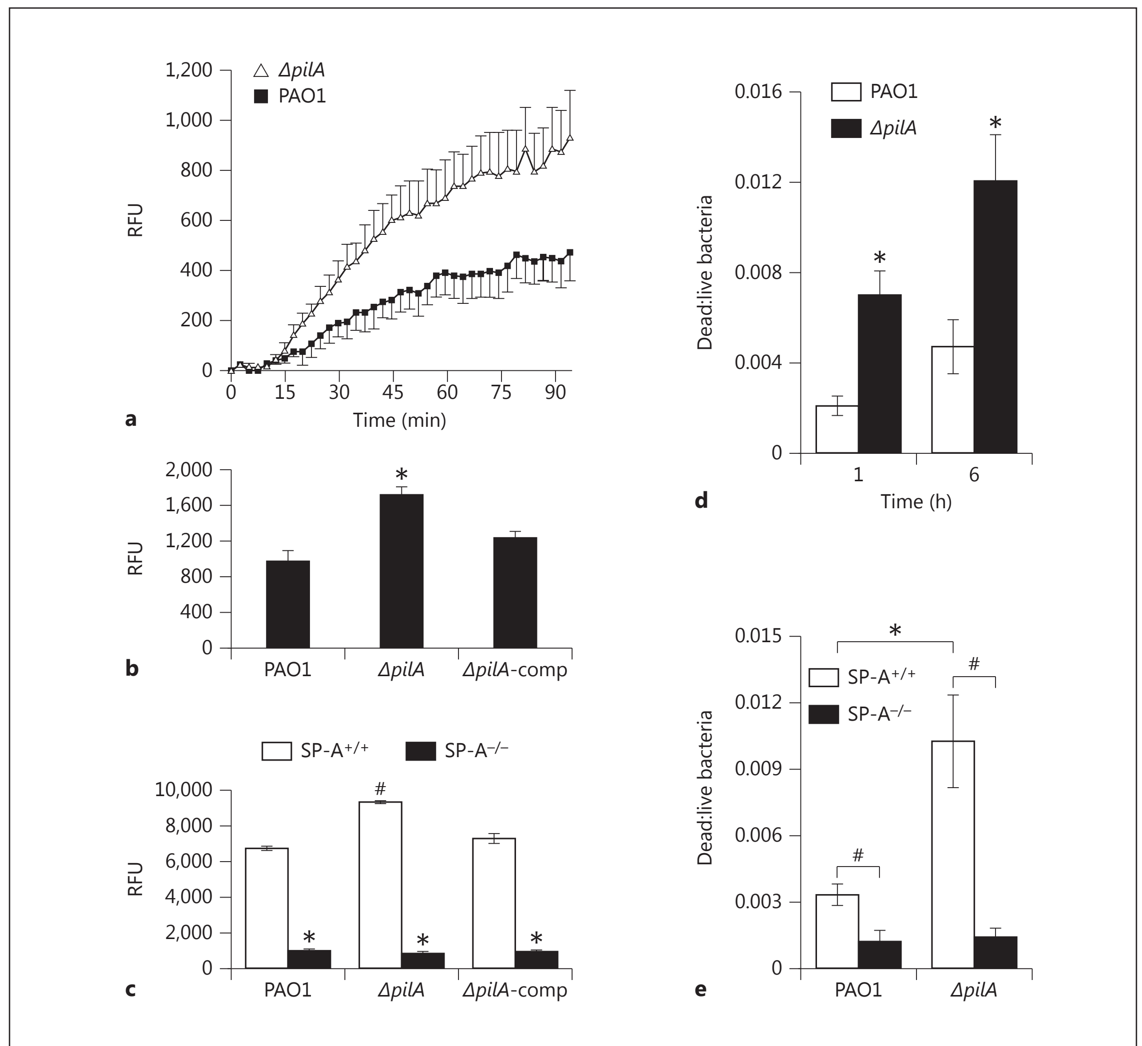
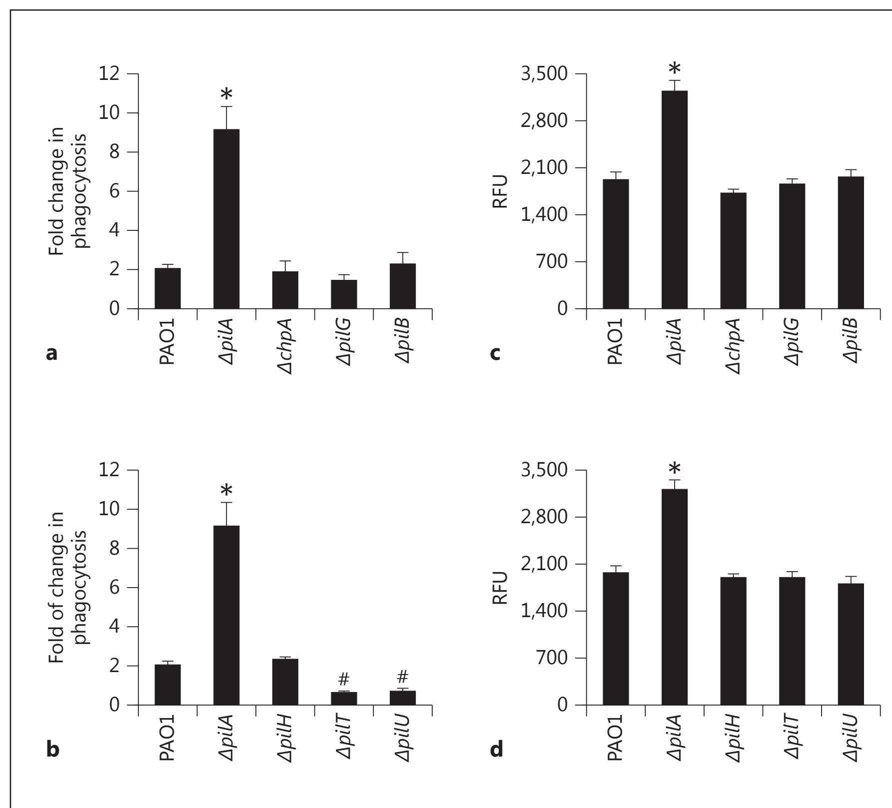


Fig. 5. *ΔpilA* are more susceptible to SP-A-mediated membrane permeabilization. **a** ELF-97 assay. PAO1 and *ΔpilA* were preincubated with 50 μg/ml hSP-A for 15 min before the addition of ELF-97. Absorbance was measured every 3 min at excitation wavelength 355 nm and emission wavelength 535 nm, for a total of 90 min. **b** In vitro ThioGlo assay. PAO1, *ΔpilA* and *ΔpilA*-comp were preincubated with 50 μg/ml hSP-A for 15 min. The bacteria-free supernatants were then mixed with ThioGlo. Absorbance was measured at excitation wavelength 405 nm and emission wavelength 535 nm. * $p < 0.05$ when comparing the relative fluorescence unit (RFU) of *ΔpilA* to PAO1 and *ΔpilA*-comp. (**c**) Ex vivo ThioGlo assay. PAO1, *ΔpilA* and *ΔpilA*-comp were preincubated with 50 μg/ml total protein of BALF from either SP-$A^{+/+}$ mice or SP-$A^{-/-}$ mice for 1 h. The bacteria-free supernatants were then mixed with ThioGlo. Absorbance was measured at excitation wavelength 405 nm and emission wavelength 535 nm. # $p < 0.05$ when comparing the RFU of *ΔpilA* to PAO1 and *ΔpilA* in BALF from SP-$A^{+/+}$ mice. * $p < 0.05$ when comparing the RFU of each *PA* strain in BALF from SP-$A^{+/+}$ to SP-$A^{-/-}$ mice. **d** In vitro *PA* killing by hSP-A. PAO1 and *ΔpilA* were preincubated with 50 μg/ml hSP-A for 1 or 6 h. The bacteria were then stained with a mixture of SYTO 9 and propidium iodide for 15 min. The ratio of dead-to-live bacteria is counted in 10 high-power fields. * $p < 0.05$ when comparing the ratio of dead-to-live *ΔpilA* to PAO1 bacteria in the presence of hSP-A. **e** Ex vivo *PA* killing by mouse SP-A within BALF. PAO1 and *ΔpilA* were preincubated with 50 μg/ml total protein of BALF from either SP-$A^{+/+}$ mice or SP-$A^{-/-}$ mice for 1 h. Bacterial viability was determined as in **d**. # $p < 0.05$ when comparing the ratio of dead-to-live bacteria between those treated with BALF from SP-$A^{+/+}$ and SP-$A^{-/-}$ mice. * $p < 0.05$ when comparing the ratio of dead-to-live PAO1 to *ΔpilA*-treated with BALF from SP-$A^{+/+}$ mice. Experiments were performed independently in triplicate (for ELF-97 and ThioGlo assays) or duplicate (for the LIVE/DEAD assay). Data from 1 typical experiment are shown.

Fig. 6. The susceptibility of Tfp extension and retraction mutants to SP-A-mediated opsonization. **a**, **b** RAW 264.7 macrophages were infected with PAO1, *ΔpilA* and the extension or retraction mutants in the presence or absence of hSP-A. The ratio of ingested bacteria between those exposed to hSP-A to those unexposed was expressed as fold increase in phagocytosis. **a** Phagocytosis of PAO1, *ΔpilA* and extension mutants *ΔchpA*, *ΔpilG* and *ΔpilB*. **b** Phagocytosis of PAO1, *ΔpilA* and retraction mutants *ΔpilH*, *ΔpilT* and *ΔpilU*. * $p < 0.05$ when comparing phagocytosis of PAO1 to the *ΔpilA* mutant. # $p < 0.05$ when comparing phagocytosis of PAO1 to the *ΔpilT* and *ΔpilU* mutants. All experiments were independently performed in triplicate. The mean ± standard deviation from 1 representative experiment is shown. **c**, **d** Membrane permeabilization of *PA* strains by hSP-A. **c** Membrane permeabilization of PAO1, *ΔpilA* and extension mutants *ΔchpA*, *ΔpilG* and *ΔpilB*. **d** Membrane permeability of PAO1, *ΔpilA* and retraction mutants *ΔpilH*, *ΔpilT* and *ΔpilU*. All experiments were independently performed in triplicate. The mean ± standard deviation from 1 representative experiment is shown.



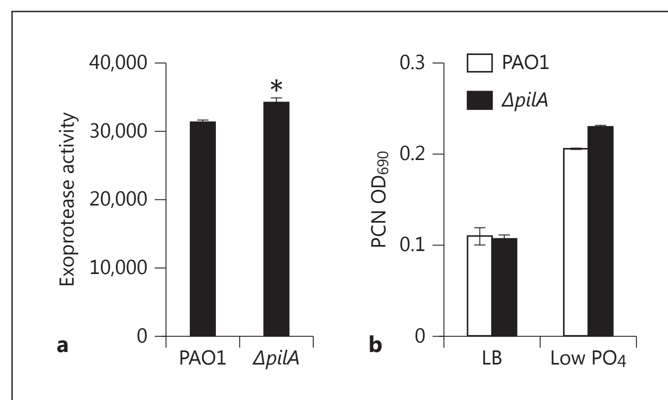


Fig. 7. Resistance to SP-A-mediated phagocytosis is independent of quorum sensing. **a** Exoprotease activity of PAO1 versus *ΔpilA*. **b** Pyocyanin production of PAO1 versus *ΔpilA*.

capable of directly killing bacteria by membrane permeabilization [17–19]. We examined the susceptibility of *ΔpilA* to SP-A-mediated membrane permeabilization, by measuring the diffusion of an impermeable phosphatase substrate ELF-97 into *PA* cells, and by measuring the leakage of thiol-containing proteins. After 90 min of exposure, the ELF-97 assay indicated that *ΔpilA* bacteria were permeabilized 2.1-fold higher than the PAO1 (fig. 5a). In addition, the leakage of thiol-containing proteins increased by 1.81-fold relative to PAO1 (fig. 5b). To confirm our in vitro observations, we compared the ability of BALF of SP-$A^{+/+}$ and SP-$A^{-/-}$ mice to permeabilize the membrane of PAO1 and *ΔpilA* (fig. 5c). The *ΔpilA* bacteria were more susceptible to BALF from SP-$A^{+/+}$ mice than PAO1. In contrast, BALF from SP-$A^{-/-}$ only possessed low levels of membrane-permeabilizing capability, suggesting that SP-A is an important membrane-permeabilizing protein within the BALF. To determine whether increased susceptibility to membrane permeability leads to direct bacterial killing, we examined the bacterial viability after exposure to hSP-A and to BALF. Although both the hSP-A and the BALF from SP-$A^{+/+}$ mice only caused low levels of *PA* killing, the *ΔpilA* was consistently shown to be significantly more susceptible to direct killing by hSP-A (fig. 5d) and BALF of SP-$A^{+/+}$ mice (fig. 5e). These results suggest that SP-A-mediated membrane permeabilization contributes minimally to *PA* clearance.

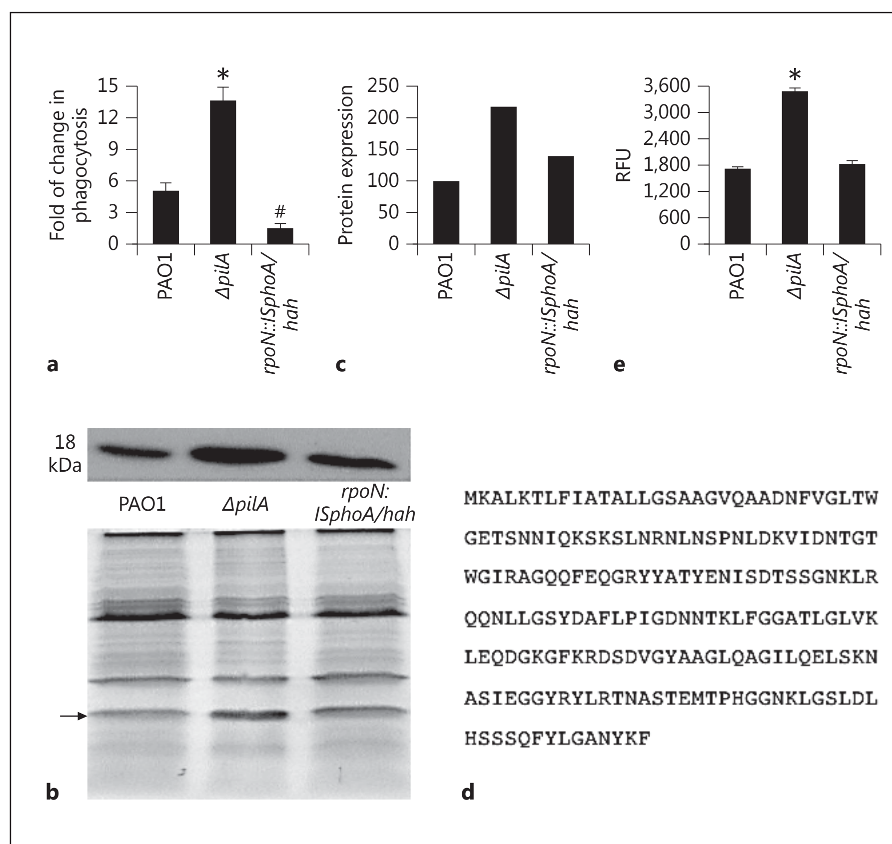


Fig. 8. Resistance to SP-A-mediated phagocytosis is regulated by RpoN. **a** Comparison of bacterial phagocytosis by RAW 264.7 macrophages between PAO1, *ΔpilA* and *rpoN::ISphoA/hah* in the presence or absence of 25 μg/ml hSP-A. All experiments were independently performed in triplicate. The mean ± standard deviation from 1 representative experiment is shown. * $p < 0.05$ when comparing PAO1 to *ΔpilA*. # $p < 0.05$ when comparing PAO1 to *rpoN::ISphoA/hah*. **b** Ligand blot analysis of nonpilus adhesins in PAO1, *ΔpilA* and *rpoN::ISphoA/hah*. **c** Image J analysis of the ligand blot. Protein expression was normalized to PAO1 as 100%. **d** Protein sequence of OprH. **e** Comparison of membrane permeabilization between PAO1, *ΔpilA* and *rpoN::ISphoA/hah*. The experiments were independently performed in triplicate. The mean ± standard deviation from 1 representative experiment is shown. * $p < 0.05$ when comparing PAO1 to *ΔpilA* and *rpoN::ISphoA/hah*.

Tfp-Mediated Resistance to SP-A-Mediated Phagocytosis Is Independent of the Presence of the Appendages on the Cell Surface

Compared to other *PA* mutants we have studied previously [15, 16, 27, 28], *ΔpilA* is uniquely more susceptible to both SP-A-mediated opsonization and membrane permeabilization. We used both the pilin extension *(ΔchpA*, *ΔpilG* and *ΔpilB)* and retraction *(ΔpilH*, *ΔpilT* and *ΔpilU)* mutants to determine if the presence of Tfp on the cell surface of *PA* is required for resistance to both SP-A-mediated opsonization and membrane permeabilization. As shown in figure 6a, the mere absence of Tfp on the bacterial surface in the extension mutants *(ΔchpA*, *ΔpilG* and *ΔpilB)* does not increase susceptibility to SP-A-mediated phagocytosis. Interestingly, the retraction mutants, *ΔpilT* and *ΔpilU*, which are hyperpiliated, are more resistant to SP-A-mediated opsonization (fig. 6b). Both extension and retraction mutants were as resistant to SP-A-mediated membrane permeabilization as PAO1 (fig. 6c, d). Collectively, these results suggest that the presence of pilin anchoring the membranes is adequate to confer resistance to SP-A-mediated phagocytosis. In addition, expression of additional Tfp (e.g. *ΔpilT* and *ΔpilU* mutants) offers increased resistance to SP-A-mediated phagocytosis.

Increased Susceptibility of ΔpilA to SP-A-Mediated Phagocytosis Is Probably Due to a Compensatory Increase in Nonpilus Adhesins

Previous work in our laboratory has shown that *PA* confers resistance to SP-A-mediated phagocytosis by increasing the degradation of SP-A through elastase B [20, 21], through mechanisms regulated by both flagellum and quorum-sensing. Flagellar-deficient mutants are deficient in quorum sensing, decreasing their ability to produce adequate elastase B to degrade SP-A. However, *ΔpilA* produced wild-type levels of both quorum-sensing-regulated exoprotease activities (fig. 7a) and the redox-active secondary metabolite pyocyanin (fig. 7b). Furthermore, the *ΔpilA* lost its susceptibility to SP-A-mediated phagocytosis after 12 h (fig. 3c), suggesting that SP-A was degraded. Thus, the lack of quorum-sensing and SP-A degradation is not the cause of enhanced opsonization of *ΔpilA* by SP-A.

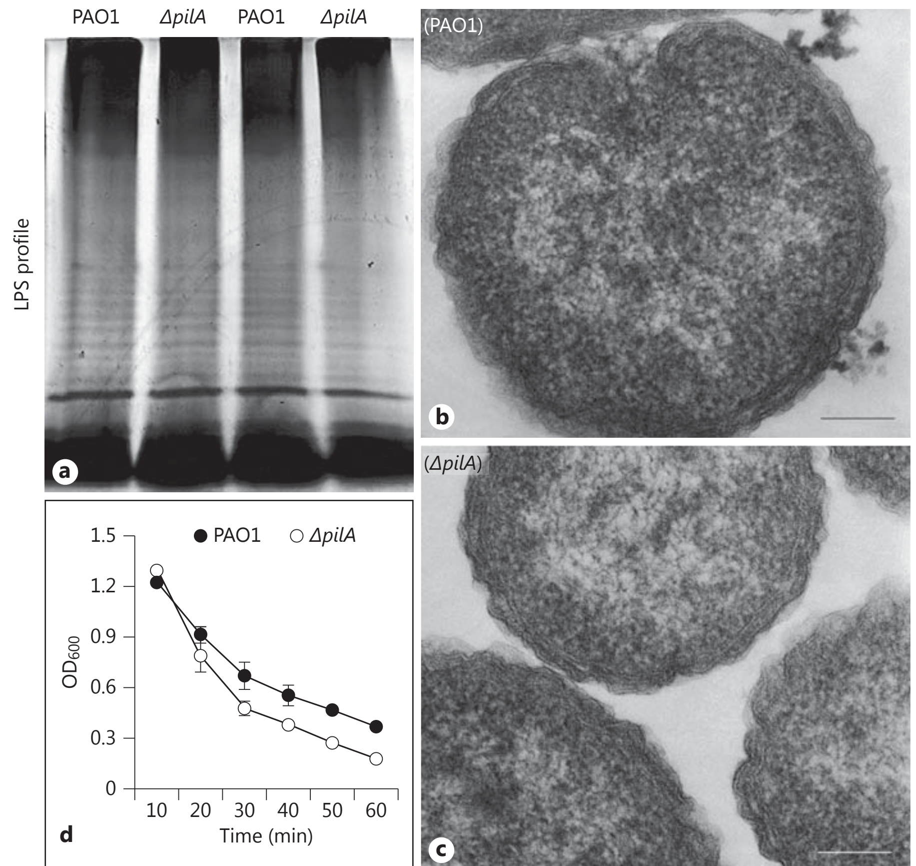


Fig. 9. *ΔpilA* is more susceptible to SDS-mediated cell lysis. **a** LPS analysis of PAO1 and *ΔpilA* as visualized using silver stain. **b**, **c** TEM of PAO1 and *ΔpilA*, respectively. Bar = 100 nm. **d** SDS lysis assay. PAO1 and *ΔpilA* bacteria were incubated in 0.25% SDS solution. OD_{600} was measured every 10 min. Bacterial lysis experiments were independently performed in triplicate. The mean from 1 representative experiment is shown.

Previous studies have shown that *ΔpilA* has a higher binding affinity to host epithelial cells than the hyperpiliated mutants *ΔpilT* and *ΔpilU* [29–32]. This is speculated to be caused by overexpression of alternative 'nonpilus adhesins' in *ΔpilA*. The alternative sigma factor RpoN has been shown to regulate the expression of these adhesins [29–32]. We hypothesized that one or more of these nonpilus adhesins may serve as ligands for binding to SP-A. We compared the phagocytosis of *ΔpilA* versus *rpoN::ISphoA/hah* in the presence of SP-A. Figure 8a shows *rpoN::ISphoA/hah*, which lacks the adhesins, is more resistant to SP-A-mediated phagocytosis than both wild-type PAO1 and *ΔpilA*. Importantly, ligand blot analysis shows that the *ΔpilA* overexpresses a protein of approximately 18 kDa compared to both PAO1 and *rpoN::ISphoA/hah* (fig. 8b, c). LCMS/MS and microsequencing analyses indicate that this putative adhesin is the outer membrane protein H1 precursor OprH (PA1178) (fig. 8d). Finally, *rpoN::ISphoA/hah* does not exhibit increased susceptibility to SP-A-mediated membrane permeabilization, suggesting that these nonpilus adhesins are dispensable against the pore-forming function of SP-A (fig. 8e).

ΔpilA Has Reduced Membrane Stability, Rendering It Susceptible to SP-A-Mediated Membrane Permeabilization

Next, we examined the potential mechanisms of increased susceptibility of *ΔpilA* to SP-A-mediated membrane permeabilization. Previously, we have shown that the loss of LPS [18, 19] and flagellum [19–21] destabilizes membrane integrity, rendering them more susceptible to SP-A-mediated membrane permeabilization. In addition, Abeyrathne et al. [33] have shown that the O-antigen ligase mutant, *ΔwaaL*, which is unable to attach O-antigen to the core polysaccharide of LPS, has absent or decreased Tfp and flagella. We examined whether the absence of Tfp destabilizes the membranes, through reduced expression of LPS. Qualitative analyses of both the LPS and the TEM images of bacterial membranes show no discernible differences between *ΔpilA* and PAO1 (fig. 9a–c). However, *ΔpilA* was more slightly more sus-

ceptible to lysis by 0.25% SDS, suggesting that the loss of pilin modestly compromises the integrity of the outer membranes in the mutant bacteria (fig. 9d).

Discussion

SP-A is a major pulmonary innate immunity protein that mediates microbial clearance through opsonization and membrane permeabilization. However, little is known about the mechanisms elaborated by microbial pathogens to confer resistance or susceptibility to SP-A. Previously, we have demonstrated that several *PA* factors: LPS, flagellum, isochorismate synthase and phosphoenolpyruvate phosphotransferase confer resistance to SP-A-mediated membrane permeabilization [18–21]. Flagellum also regulates the quorum-sensing-mediated expression of elastase B that degrades and disables SP-A-mediated opsonization during phagocytosis [19–21]. In this study, we found that Tfp of *PA* is uniquely important for resistance to SP-A-mediated opsonization and membrane permeabilization. Several lines of evidence support this argument: (1) *ΔpilA* is preferentially cleared in the lungs of SP-A$^{+/+}$ mice compared to SP-A$^{-/-}$ mice, (2) *ΔpilA* is more susceptible to SP-A-mediated aggregation and opsonization, (3) *ΔpilA* is more susceptible to SP-A-mediated membrane permeabilization and (4) the genetically complemented *ΔpilA*-(comp strain, which carries a copy of the wild-type *pilA* gene in trans, has restored resistance to SP-A-mediated opsonization and membrane permeabilization.

At face value, our report seems to contradict previous findings, which show that Tfp-deficient *PA* strains, including *ΔpilA*, are more resistant to phagocytosis by macrophages [34–36]. However, this discrepancy can be explained because of different experimental context. Firstly, these previously published studies were pertaining to nonopsonin-mediated, fibronectin-dependent phagocytosis. Secondly, the number of phagocytized bacteria was quantified using a semi-quantitative fluorescent technique, rather than the quantitative method we used with the gentamicin exclusion assay. However, the gentamicin exclusion assay, which has been used for decades to quantify microbial phagocytosis, has its disadvantages. Because phagocytes ingest both individual and SP-A-aggregated bacteria, we cannot rule out the possibility that the vigorous pipetting and vortexing procedures used during serial dilution plating may not completely disaggregate bacteria. However, even if this were the case, *ΔpilA*, which is more susceptible than PAO1 to SP-A-mediated aggregation, would be undercounted. This would mean that the actual difference in phagocytic factor between *ΔpilA* and PAO1 would be even higher, further supporting our results showing that *ΔpilA* is more susceptible to SP-A-mediated opsonization and phagocytosis.

A careful examination of data presented in figure 3 (a, b) indicates that there is variation in the efficiency of phagocytosis in different sets of experiments. This is not unusual, due to the fact that bacteria and macrophage cultures are not 'identical' from one set of experiments to the next; for example, the number of bacterial cells used in each experiment certainly differs slightly. Another contributing factor to the variation in phagocytic efficiency is that different batches of SP-A preparations derived by lung washing from different alveolar proteinosis patients may have different antimicrobial activity and potency. Nevertheless, in each experiment described, *ΔpilA* is consistently more susceptible to SP-A-mediated opsonization and membrane permeabilization.

Detailed genetic analyses of the *PA* mutants defective in retraction and extension of Tfp reveal rather unexpected mechanisms by which the pilus mediates resistance to SP-A-mediated phagocytosis. For example, the extension gene mutants *(ΔchpA*, *ΔpilG* and *ΔpilH)* did not show increased susceptibility to SP-A-mediated phagocytosis. These results suggest that a total absence of *pilA* both intracellularly and extracellularly is required to render *PA* becoming susceptible to SP-A-mediated opsonization. We postulate that the absence of *pilA* causes a compensatory increase of alternative ligands that actually interact with SP-A. This is supported by previous studies showing that Tfp-deficient *PA* mutants (e.g. *ΔpilA*) have higher adhesion to epithelial cells than hyperpiliated Tfp extension mutants strains *ΔpilT and ΔpilU*, suggesting that Tfp-independent adhesins are responsible for increased binding to epithelial cells [7, 29]. Importantly, both hyperpiliated Tfp mutants *ΔpilT and ΔpilU* are significantly more resistant than the wild-type PAO1 to SP-A-mediated phagocytosis. These observations suggest that additional Tfp expressed by both *ΔpilT and ΔpilU* mutants may have masked the putative ligands, decreasing SP-A-mediated binding, aggregation and subsequent opsonization, and enhanced phagocytosis by phagocytes.

Previous work has shown that the alternative sigma factor, RpoN, positively regulates both pilin and flagellin production through their respective 2-component systems *PilRS* and *FleQR* [32, 37]. Thus, RpoN deficiency results in the loss of both Tfp and flagellum in *PA*. Interestingly, *ΔpilA* has been used as a model to study the expression of alternative nonpilus adhesins. In contrast,

rpoN mutation has been used as a model for the lack of nonpilus adhesins [30–32]. We speculate that the absence of Tfp causes RpoN to magnify the production of nonpilus adhesins, which serve as ligands for SP-A. This argument is supported by our analyses showing that *ΔrpoN* is even more resistant to SP-A-mediated phagocytosis than PAO1. Several studies have tried to determine the identity of these nonpilus adhesins, with most of them focusing on those that bind mucins [27, 28, 38, 39]. Other studies focus on the role of nonpilus adhesins in nonopsonic phagocytosis [30, 31, 39]. However, the identity of these adhesins remains elusive. Another complication is the uncertainty whether the alternative adhesins associated with binding to mucin are the same as those conferring susceptibility to SP-A-mediated phagocytosis. By using ligand blot, we have identified a putative non-pilus adhesin that binds SP-A. LC-MS/MS and micro-sequencing analyses show that the SP-A ligand is the 18-kDa outer membrane protein H1 precursor OprH. OprH expression is governed by low-magnesium environment through the PhoP-PhoQ two-component regulatory system [40, 41]. Interestingly, OprH is noted to have direct interaction with LPS, which is bound by the CRD of SP-A [40]. Another outer membrane protein, P2 of *Hemophilus influenzae*, has been shown to be a receptor of SP-A [42]. We are currently constructing nonpolar deletion mutant and study the genetic, molecular and cellular interactions of OprH with Tfp, RpoN, and SP-A. Furthermore, we are examining the sequence and functional homology between OprH and *H. influenzae* P2.

Tfp interacts with glycoconjugates (e.g. asialoGM1) on the host epithelium [4]. Similarly, SP-A also interacts with glycoconjugates through its CRD. Thus, another possible mechanism of preferential clearance of *ΔpilA* mutant from mouse lungs is that SP-A may have blocked Tfp receptors, and preventing the binding of *PA* to the airway epithelium of wild-type mice. In contrast, in SP-$A^{-/-}$ mice, these Tfp receptors will be exposed, allowing better binding and colonization of PA to the lungs devoid of SP-A. Future studies will include performing comparative in vivo binding of wild-type versus various Tfp mutants to the lung epithelium of SP-$A^{+/+}$ versus SP-$A^{-/-}$ mice.

Our experimental evidence demonstrates that Tfp also mediates resistance to SP-A-mediated membrane permeabilization. Following exposure to hSP-A and to the BALF from SP-$A^{+/+}$ mice, the integrity of the outer membranes is compromised, rendering the *ΔpilA* bacteria more permeable to the phosphatase substrate ELF-97 into the cells and increased leakage of thiol-containing intracellular proteins out of the cells. Previously, we have shown the the wild-type strain PAO1 is highly resistant to direct killing by SP-A. Although *ΔpilA* is more susceptible to SP-A-mediated membrane permeabilization, this only results in small increase in direct killing of the bacteria, suggesting that the pore-forming process may be transient. Qualitative LPS analysis and TEM examination of membrane structures did not reveal any gross alterations in *ΔpilA*. However, *ΔpilA* bacteria have reduced membrane stability as demonstrated by a modest increase of cell lysis by 0.25% SDS.

In conclusion, we have shown that Tfp is important for resistance to SP-A-mediated opsonization and membrane permeabilization. We provide evidence that Tfp may camouflage nonpilus adhesin, and prevent the binding of SP-A to these ligands, reducing the opsonization and phagocytosis by macrophages. In addition, Tfp is necessary to stabilize bacterial membranes, rendering *PA* more resistant to SP-A-mediated membrane permeabilization. Adjunctive treatment regimen aimed at inhibiting Tfp may help to improve the clearance of *PA* by augmenting the efficiency of SP-A in killing this important respiratory pathogen.

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