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Type IV Pilus Glycosylation Mediates Resistance of *Pseudomonas aeruginosa* to Opsonic Activities of the Pulmonary Surfactant Protein A

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Pseudomonas aeruginosa **is a major bacterial pathogen commonly associated with chronic lung infections in cystic fibrosis (CF). Previously, we have demonstrated that the type IV pilus (Tfp) of *P. aeruginosa* mediates resistance to antibacterial effects of pulmonary surfactant protein A (SP-A). Interestingly, *P. aeruginosa* strains with group I pilins are *O*-glycosylated through the TfpO glycosyltransferase with a single subunit of O-antigen (O-ag). Importantly, TfpO-mediated *O*-glycosylation is important for virulence in mouse lungs, exemplified by more frequent lung infection in CF with TfpO-expressing *P. aeruginosa* strains. However, the mechanism underlying the importance of Tfp glycosylation in *P. aeruginosa* pathogenesis is not fully understood. Here, we demonstrated one mechanism of increased fitness mediated by *O*-glycosylation of group 1 pilins on Tfp in the *P. aeruginosa* clinical isolate 1244. Using an acute pneumonia model in SP-A$^{+/+}$ versus SP-A$^{-/-}$ mice, the *O*-glycosylation-deficient Δ*tfpO* mutant was found to be attenuated in lung infection. Both 1244 and Δ*tfpO* strains showed equal levels of susceptibility to SP-A-mediated membrane permeability. In contrast, the Δ*tfpO* mutant was more susceptible to opsonization by SP-A and by other pulmonary and circulating opsonins, SP-D and mannose binding lectin 2, respectively. Importantly, the increased susceptibility to phagocytosis was abrogated in the absence of opsonins. These results indicate that *O*-glycosylation of Tfp with O-ag specifically confers resistance to opsonization during host-mediated phagocytosis.**

Pseudomonas aeruginosa is one of the most common causes of nosocomial infections in humans, especially chronic infection in patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (1–3). It is also a primary cause of death and sepsis in immunocompromised individuals (4, 5). Antibiotic-resistant *P. aeruginosa* is a serious clinical problem that can lead to denial for lung transplant, infection, and death (6). Thus, there is an urgent need to explore alternative strategies, including the possibility of augmenting the expression of pulmonary innate immunity proteins to manage *P. aeruginosa*-mediated infections.

Bacterial type IV pilus (Tfp) is important for multiple cellular functions, including surface motility, microcolony and biofilm formation, host-cell adhesion, cell signaling, DNA uptake by natural transformation, and phage attachment (7). Tfp is also an important virulence factor of *P. aeruginosa* (8). *P. aeruginosa* can be separated into five groups based on the presence or absence of varying downstream accessory genes flanking the pilin gene, *pilA* (9, 10). The PilA pilins of these five *P. aeruginosa* groups have different amino acid sequences, lengths, and presence of posttranslational modifications (9–13). The downstream accessory genes function in either pilin posttranslational glycosylation (group I and group IV) or modulation of pilus assembly (group III and group V) (9, 10, 14, 15).

Group I alleles (e.g., strain 1244), which contain a glycosyltransferase (PilO/TfpO) that glycosylates each pilin of Tfp with one subunit of O-antigen (O-ag), are frequently associated with CF and environmental isolates (10). The O-ag repeating subunit was noted to be the trisaccharide pseudaminic acid (5NβOHC$_4$7NFmPse)-(2→4)-xylose-(1→3)-*N*-acetylfucosamine (FucNAc), bound to serine residue 148 at the carboxyl terminal of the pilin (16, 17). The O-ag glycan decorating PilA is the product of the same O-ag biosynthetic pathway for the lipopolysaccharide (LPS) O-ag of the same strain (18). Group II alleles, which do not contain an accessory flanking gene, include common laboratory strains PAO1 and PAK (10). Group III alleles contain the accessory gene *tfpY* and include the human clinical isolate PA14 (10). PA14 was shown to produce a lower 50% lethal dose (LD$_{50}$) and a higher mortality rate than PAO1 (19). This difference in virulence was partially attributed to *ybtQ*, a gene present in PA14 but not PAO1 (19). Also, PA14 contains the pathogenicity islands PAPI-1 and PAPI-2, which have been shown to contribute to virulence (20, 21). Group IV alleles contain two accessory genes, *tfpW* and *tfpX* (10). The PilA pilins of group IV alleles are glycosylated with a homo-oligomer of α-1,5-linked D-arabinofuranose, which is similar to the lipoarabinomannan polymer found in the cell wall of *Mycobacterium* spp. (12). Group V alleles contain the accessory TfpZ gene (10). TfpY of group III alleles and TfpZ of group V alleles have been shown to be important for the surface expression of PilA (14).

Most recently, we have demonstrated that Tfp of *P. aeruginosa* mediates resistance to antibacterial effects of pulmonary surfactant protein A (SP-A), an important innate immunity protein that

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TABLE 1 Bacterial strains and plasmids used in this study

P. aeruginosa strain	Description	Source or reference
1244	Wild type	11
1244G7 (Δ*tfpO*)	Nonpolar gene replacement of the *tfpO* gene in the wild-type strain 1244 with a gentamicin cassette	11
1244G7-comp (Δ*tfpO*-comp)	Δ*tfpO* mutant complemented with a copy of wild-type gene in *trans*	11
1244Δ*fgtA*	Nonpolar replacement of the *fgtA* gene in the wild-type strain 1244 with a gentamicin cassette	This study
PAO1	Wild type	23, 44
PAO1Δ*fgtA*	Nonpolar replacement of the *fgtA* gene in the wild-type strain PAO1 with a gentamicin cassette	23, 44
PAK	Wild type	23, 44
PAKΔ*fgtA*	Nonpolar replacement of the *fgtA* gene in the wild-type strain PAK with a gentamicin cassette	23, 44

induces opsonization and membrane permeability in microbes (22). Despite reports showing frequent association of *P. aeruginosa* strains in the TfpO-expressing group I alleles with CF and the importance of TfpO-mediated glycosylation as a virulence factor in mouse lung infection (10, 11), the molecular basis behind the importance of Tfp glycosylation in *P. aeruginosa* pathogenesis, especially in the context of alveolar epithelium, is not fully understood. In the present study, we examined the mechanisms by which Tfp glycosylation with O-ag contributes to lung infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The clinical *P. aeruginosa* strain 1244, the TfpO-deficient mutant strain 1244G7 (Δ*tfpO* strain), and the genetically complemented strain (Δ*tfpO*-comp strain) were generously provided by Peter Castric (Duquesne University) (11) (Table 1). Laboratory wild-type strains PAO1 and PAK and their Δ*rfbC* mutants (Table 1) were generous gifts from Reuben Ramphal (University of Florida). The Δ*rfbC* mutant was constructed in strain 1244 by gene replacement with a gentamicin resistance gene cassette as described previously (23) (Table 1). Strain 1244 was grown in plain Luria-Bertani (LB) broth (Fisher Scientific); the Δ*tfpO* and Δ*rfbC* strains were grown in LB broth containing 75 μg of gentamicin (Life Technologies)/ml, and the Δ*tfpO*-comp strain was grown in LB medium containing 200 μg of carbenicillin (Lab Scientific)/ml at 37°C overnight. The strains were then stored at −80°C in 25% glycerol (Sigma-Aldrich). Before each experiment, bacteria were streaked from frozen stock onto LB agar with or without antibiotic for 18 h at 37°C. One colony from this streak was then cultured in 5 ml of LB broth to stationary phase to an optical density at 600 nm of ~3.0 by using a Genesys 10 UV spectrophotometer (Thermo Scientific).

Mouse infection assays. Wild-type C3H/HeN (SP-$A^{+/+}$) mice were purchased from Harlan Laboratories (Indianapolis, IN). Isogenic SP-$A^{-/-}$ mice were gifts from Francis McCormack (University of Cincinnati College of Medicine). Animal studies were carried out in strict accordance with the protocol approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. SP-$A^{+/+}$ and SP-$A^{-/-}$ mice (groups of 10) were given a single intranasal inoculation of 3×10^6 CFU of the parental clinical strain 1244 or the Δ*tfpO* mutant. After 18 h, mouse lungs were harvested for histology ($n = 3$) or bacterial enumeration ($n = 7$) as previously described (22, 24). For mortality studies, animals ($n = 10$) were monitored for up to 48 h. Moribund animals that displayed rough hair coat, hunched posture, distended abdomen, lethargy, or inability to eat or drink were euthanized and counted as dead.

***In vivo* phagocytosis assays.** The phagocytosis rates between different *P. aeruginosa* strains were compared using a modified gentamicin exclusion assay as previously described (22, 24). Briefly, C3H/HeN and C3H SP-$A^{-/-}$ mice ($n = 3$) were given a single intranasal inoculation of 10^7 CFU of 1244 or Δ*tfpO* strain. After 90 min, mouse lungs were lavaged to collect the alveolar macrophages and neutrophils. The white blood cells were then incubated in phosphate-buffered saline (PBS) with 100 μg of piperacillin (Sigma-Aldrich)/ml to kill the remaining extracellular bacteria (25). Piperacillin was used instead of gentamicin because the Δ*tfpO* mutant contains a gentamicin resistance cassette (11). The macrophages were lysed with 1% Triton X-100 solution (Fisher Scientific) and serially diluted for *P. aeruginosa* CFU enumeration. The ratio of CFU counts between C3H/HeN and C3H SP-$A^{-/-}$ mice was computed for the fold increase of phagocytosis mediated by SP-A. The changes in phagocytosis were then normalized to 1244, which was set to the baseline value of 1.

Purification of hSP-A. Discarded lung washings from anonymous alveolar proteinosis patients were generously provided by Francis McCormack (University of Cincinnati College of Medicine). Pulmonary alveolar proteinosis is a rare lung disease with abnormally high accumulation of surfactant proteins that occurs within the alveoli (26). Human SP-A (hSP-A) was purified as previously described (27). Briefly, raw lung washings, equilibrated with 1 mM $CaCl_2$ (Sigma-Aldrich), were passed through a Sepharose 6B column (GE Healthcare) laden with mannose (Sigma-Aldrich). The captured SP-A was then eluted using elution buffer (2 mM EDTA, 5 mM Tris-HCl [pH 7.4]). The eluted fractions were dialyzed using the dialysis buffer (150 mM NaCl, 5 mM Tris-HCl [pH 7.4]) to remove the EDTA. The purity of hSP-A preparations was confirmed by Coomassie blue analysis.

Murine macrophage culture and *in vitro* phagocytosis assays. Murine RAW 264.7 macrophages (ATCC TIB-71) were maintained in Dulbecco modified Eagle medium (DMEM; Corning) supplemented with 10% fetal bovine serum (Phoenix Research Products) at 5% CO_2 and 37°C (28). The phagocytosis rates between different *P. aeruginosa* strains were compared using the modified gentamicin exclusion assay. Briefly, 10^6 RAW 264.7 macrophages/ml were plated in six-well cell culture plates overnight at 37°C and 5% CO_2. For dose-dependent SP-A-mediated phagocytosis, *P. aeruginosa* strains were preincubated with 12.5 or 25 μg of hSP-A/ml in the presence of 2 mM $CaCl_2$ for 1 h in a rotating incubator at 37°C. For time-dependent SP-A-mediated phagocytosis, *P. aeruginosa* strains were preincubated with 25 μg of hSP-A/ml in the presence of 2 mM $CaCl_2$ for 1, 6, or 12 h in a rotating incubator at 37°C. The resulting mixture was then incubated with the RAW 264.7 cells at a ratio of 10 bacteria to 1 macrophage for 90 min. The macrophages were then washed and incubated with DMEM with 100 μg of piperacillin/ml to kill the remaining extracellular bacteria. The macrophages were lysed with 1% Triton X-100 solution, and internalized bacteria were 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. The changes in phagocytosis were then normalized to strain 1244, which was set to the baseline value of 1.

Opsonic phagocytosis assays with pulmonary opsonin SP-D (Novoprotein, catalog no. C541) and circulatory opsonin mannose-binding lectin 2 (MBL-2; Novoprotein, catalog no. C488) were performed by preincubating *P. aeruginosa* strains with the opsonins in a rotating incubator at 37°C for 1 h before exposure to RAW 264.7 macrophages. Nonopsonic phagocytosis studies were performed with the same volume of sterile PBS as a substitute for opsonins.

Membrane permeabilization assays. Membrane permeabilization effects of hSP-A on *P. aeruginosa* were observed using the thiol-specific fluorophore ThioGlo (Calbiochem), as previously described (28, 29). Stationary-phase *P. aeruginosa* bacteria were washed and incubated with 50 μg of hSP-A/ml for 15 min at 37°C. For the ThioGlo assay, bacterium/SP-A mixture was sedimented, and the bacterium-free supernatant was incubated with 10 μM ThioGlo reagent. Fluorescence was measured using an excitation wavelength of 405 nm and an emission wavelength of 535 nm. The fluorescence measurement was read using a SpectraMax Gemini EM spectrophotometer (Molecular Devices).

Statistical analysis. Quantitative data were expressed as the means ± the standard errors. Statistical significance comparisons for samples with equal variances were determined by using a parametric Student *t* test for two unpaired samples. To compare the means of groups of three or more, data were analyzed for statistical significance by the one-way analysis of variance (ANOVA), followed by Tukey's tests for comparison between the means. Comparison of mouse mortality was performed by using the Fisher exact test. A significant difference was considered to be $P < 0.05$.

RESULTS

Glycosylation of Tfp with O-ag is important for resistance to SP-A-mediated lung clearance. To determine the contribution of O-ag on Tfp in resistance to SP-A-mediated clearance, we compared the lung infection by the wild-type *P. aeruginosa* 1244 and the isogenic glycosyltransferase-deficient Δ*tfpO* mutant in a mouse model of acute pneumonia. At 18 h after intranasal inoculation with 1244, SP-A$^{+/+}$ mice showed no mortality, while SP-A$^{-/-}$ mice showed a 7/10 mortality. In contrast, the Δ*tfpO* mutant caused no mortality in SP-A$^{+/+}$ mice but only a 1/10 mortality in the SP-A$^{-/-}$ mice (Fig. 1A). The number of viable Δ*tfpO* bacteria in SP-A$^{-/-}$ mice was significantly higher (2.39 logs) than in SP-A$^{+/+}$ mice. In contrast, the burden of 1244 bacteria was only 1 log higher in the SP-A$^{-/-}$ mice than in the SP-A$^{+/+}$ mice (Fig. 1B). The 1244 and Δ*tfpO* mutant strains showed comparable growth rates, indicating that attenuation in the lungs of mice infected with the latter was not due to potential growth defects caused by the gene deletion (Fig. 1C). These data suggest that the presence of *O*-glycosylation of Tfp with O-ag allows for increased resistance to SP-A-mediated lung clearance.

The aforementioned virulence studies were further supported by histopathological analysis of infected lungs (Fig. 2). The parental clinical strain 1244 caused moderate bronchopneumonia (Fig. 2A), whereas the Δ*tfpO* mutant caused mild bronchopneumonia (Fig. 2B) in SP-A$^{+/+}$ mice. In contrast, 1244 caused severe bronchopneumonia (Fig. 2C), whereas the Δ*tfpO* mutant caused only moderate bronchopneumonia in the lungs of SP-A$^{-/-}$ mice (Fig. 2D). These results indicate that *O*-glycosylation of Tfp increases the resistance of *P. aeruginosa* to SP-A-mediated lung clearance.

***O*-glycosylation of Tfp does not alter resistance of *P. aeruginosa* to SP-A-mediated membrane permeabilization.** As discussed above, SP-A mediates its antimicrobial effects by enhancing microbial clearance through membrane permeabilization and opsonization (22, 24, 28–32). To further examine whether *O*-glycosylation of *P. aeruginosa* Tfp with O-ag increases the resistance to SP-A-mediated membrane permeability, we measured the amounts of leaked thiol containing proteins from 1244 versus Δ*tfpO* mutant after exposure to hSP-A. The three 1244, Δ*tfpO*, and Δ*tfpO*-comp strains were equally susceptible to SP-A-mediated permeability (Fig. 3). These results indicate that *O*-glycosylation of Tfp does not modulate susceptibility or resistance to membrane permeabilization function of SP-A. Thus, SP-A-mediated membrane permeabilization is unlikely to contribute to preferential clearance of the Δ*tfpO* mutant strain from SP-A$^{+/+}$ lungs.

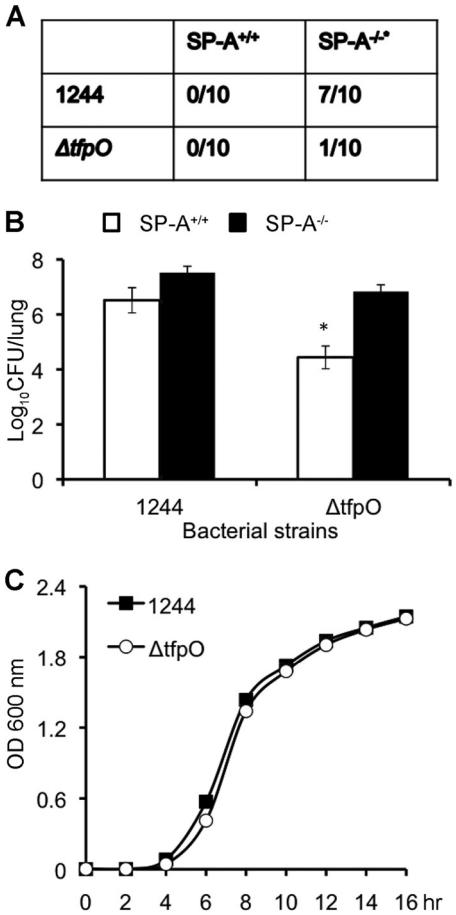

FIG 1 *O*-glycosylation of Tfp allows for increased resistance to SP-A during acute pneumonia. (A) Mortality rate of SP-A$^{+/+}$ and SP-A$^{-/-}$ mice ($n = 10$) infected with either 1244 or Δ*tfpO*. *, $P < 0.01$ when comparing the mortality rate of SP-A$^{+/+}$ versus SP-A$^{-/-}$ mice infected by strain 1244 and when comparing the mortality rate of SP-A$^{-/-}$ mice infected by 1244 versus the Δ*tfpO* mutant. Statistical analyses were performed by using the Fisher exact test. (B) Respiratory tract infections with wild-type strain 1244 versus the Δ*tfpO* mutant were performed by intranasal inoculation of anesthetized SP-A$^{+/+}$ or SP-A$^{-/-}$ mice. Mouse lungs were harvested 18 h postinfection for bacterial enumeration. The data are the mean CFU ± the standard errors (SE; $n = 7$ per group). Statistical significance comparisons among various groups were determined by using one-way ANOVA ($P < 0.01$). *, $P < 0.01$ when comparing the bacterial loads between the 1244 strain versus the Δ*tfpO* mutant infecting SP-A$^{+/+}$ mice, between the 1244 strain versus the Δ*tfpO* mutant infecting SP-A$^{-/-}$ mice versus SP-A^{+}/$^{+}$ mice, and between SP-A$^{+/+}$ mice versus SP-A$^{-/-}$ mice infected by the Δ*tfpO* mutant determined using Tukey's test. (C) The growth kinetics of 1244 and Δ*tfpO* bacteria were determined by measuring optical density at 600 nm. The experiments were performed three times independently in triplicates. The representative growth curve from one of three independent experiments is shown.

***O*-glycosylation of Tfp increases the resistance of *P. aeruginosa* to SP-A-mediated phagocytosis.** Next, we examined whether the Δ*tfpO* bacteria were more susceptible to SP-A-mediated opsonization. In the presence of 12.5 and 25 μg of SP-A/ml, Δ*tfpO* bacteria were phagocytosed 2.5- to 3.4-fold more efficiently than the parental strain 1244 (Fig. 4A). Also, we examined the phagocytosis of Δ*tfpO* bacteria in a time-dependent manner. We found that Δ*tfpO* bacteria were more susceptible to SP-A-medi-

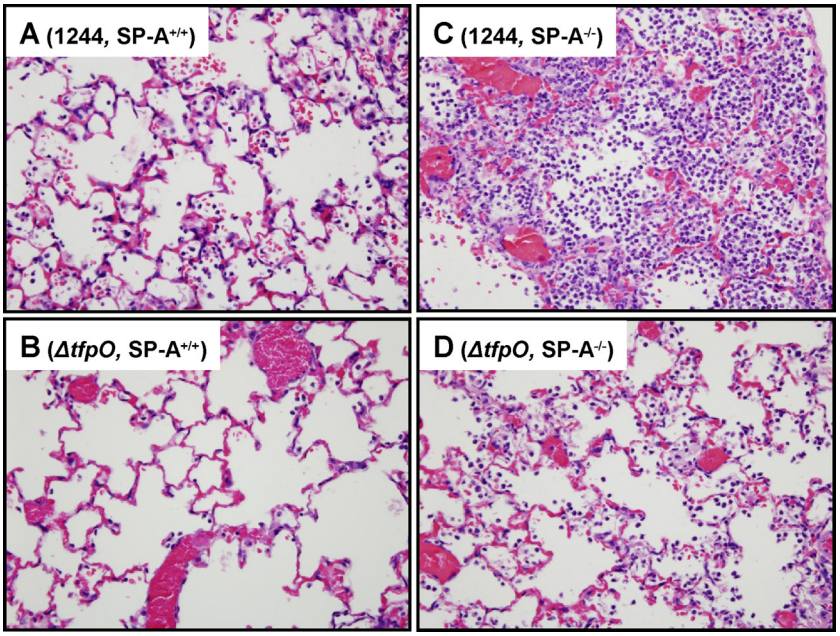

FIG 2 Histopathology of *P. aeruginosa*-infected lungs. SP-A$^{+/+}$ and SP-A$^{-/-}$ mice were infected with the 1244 strain or the Δ*tfpO* mutant as described in Fig. 1. Representative hematoxylin-eosin-stained lung sections from SP-A$^{+/+}$ and SP-A$^{-/-}$ mice ($n = 3$) at 18 h after intranasal instillation of 1244 (A and C) and Δ*tfpO* (B and D) bacteria.

ated opsonization than 1244 at 1 h after exposure to RAW 264.7 macrophages (Fig. 4B). Prolonged exposure abolished the difference of phagocytic efficiency between 1244 and Δ*tfpO* strains, most probably due to the production of exoproteases that degraded SP-A, as we had previously reported (28, 32). These observations were confirmed by *in vivo* phagocytosis assays, which showed that Δ*tfpO* bacteria were 4.5-fold more susceptible to SP-A-mediated phagocytosis than was strain 1244 (Fig. 4C). The *in vivo* phagocytosis assay measured the total phagocytosis activity involving both alveolar macrophages and neutrophils. Collectively, these results indicate that *O*-glycosylation of *P. aeruginosa* Tfp promotes resistance to opsonization mediated by host opsonins, including the pulmonary SP-A.

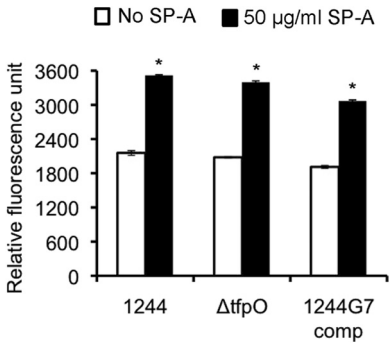

FIG 3 *O*-glycosylation of Tfp does not confer increased resistance to SP-A-mediated membrane permeabilization. An *in vitro* ThioGlo assay was performed. 1244, Δ*tfpO*, and 1244G7-comp strains were preincubated with 50 μg of hSP-A/ml for 15 min. The bacterium-free supernatants were then mixed with ThioGlo. The absorbance was measured at an excitation wavelength of 405 nm and an emission wavelength of 535 nm. *, $P < 0.05$ when comparing the relative fluorescence units of SP-A-treated bacteria versus untreated bacteria using one-way ANOVA.

***O*-glycosylation of Tfp specifically confers resistance to opsonization by both pulmonary and circulatory opsonins.** We postulated that *O*-glycosylation of Tfp with O-ag might be specifically required for resistance of *P. aeruginosa* to opsonization mediated by pulmonary and/or circulatory opsonins (30, 31, 33–36). To test this hypothesis, we examined the phagocytosis of 1244 versus Δ*tfpO* bacteria in the absence of SP-A. In contrast to data presented in Fig. 4, the uptake of 1244 bacteria was indistinguishable from that of Δ*tfpO* bacteria, suggesting that *O*-glycosylation of Tfp was specifically required for conferring resistance to opsonization by SP-A (Fig. 5A).

To confirm the findings in Fig. 4 and 5A, we examined the susceptibility of Δ*tfpO* bacteria to other opsonins. Significantly, when the 1244 and Δ*tfpO* strains were preopsonized by pulmonary surfactant protein D (SP-D) and the circulatory opsonin MBL-2, the phagocytosis of the Δ*tfpO* mutant by RAW 264.6 macrophages increased by 55 and 49%, respectively (Fig. 5B and C). Collectively, these results indicate that O-ag glycosylation of Tfp specifically confers resistance to opsonization by both pulmonary and circulatory opsonins.

Glycosylation on flagella of *P. aeruginosa* is not important for resistance to SP-A-mediated opsonization. We have previously shown that flagellum, another prominent appendage of *P. aeruginosa*, is important for resistance to SP-A-mediated opsonization and membrane permeabilization (32, 37). Similar to Tfp, the flagella of *P. aeruginosa* can be classified into two groups (a-type and b-type) based on the posttranslational *O*-glycosylation of flagellins with deoxyhexose (23, 38–44). *P. aeruginosa* strain PAK harbors the a-type *O*-glycosylated flagellins, whereas strains PAO1 and 1244 have the b-type *O*-glycosylated flagellins (41, 44). Originally named *rfbC* (23), the gene encoding the flagellar glycosyltransferase was later renamed *fgtA* (*PA1091*) (44). Flagellin glycosylation has been reported to be important for vir-

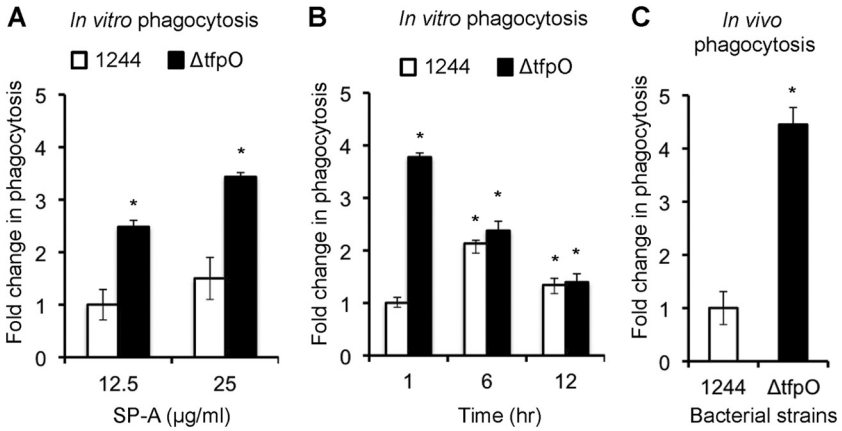

FIG 4 The Δ*tfpO* mutant is more susceptible to SP-A-mediated opsonization. RAW 264.7 macrophages were infected with either 1244 or the Δ*tfpO* mutant in the presence or absence of hSP-A. The ratio of ingested bacteria between those exposed to hSP-A and those unexposed was expressed as the fold increase in phagocytosis. The changes in phagocytosis were then normalized to 1244, which was set to the baseline value of 1. (A) Phagocytosis of the 1244 and Δ*tfpO* strains in the presence of different concentrations of hSP-A. Bacteria were preincubated with the indicated concentrations of hSP-A for 1 h prior to phagocytosis. (B) Time-dependent phagocytosis of 1244 versus the Δ*tfpO* mutant in the presence of 25 μg of hSP-A/ml. (C) *In vivo* phagocytosis of 1244 versus the Δ*tfpO* mutant. The fold changes in phagocytosis were determined in SP-A$^{+/+}$ and SP-A$^{-/-}$ mice ($n = 3$) and normalized against the phagocytosis in SP-A$^{-/-}$ mice. The changes in phagocytosis were then normalized to strain 1244, which was set to the baseline value of 1. All phagocytosis experiments were independently performed three times. The means ± the standard errors from a representative experiment are shown. *, $P < 0.05$ when comparing the numbers of phagocytosed Δ*tfpO* mutant versus 1244 by using one-way ANOVA (A and B) and a parametric Student *t* test (C).

ulence in a burn infection (42). In addition, flagellar glycosylation plays a role in colonization of the gut by *Campylobacter jejuni* 81-176 (45). Furthermore, glycosylation of flagellum in the plant-pathogenic *Pseudomonas syringae* pv. glycinea has been reported to determine the recognition of virulence by host plants, resulting in the hypersensitivity reaction to flagellin (46). Given our data showing the importance of Tfp glycosylation against opsonization, we examined the role of flagellin glycosylation in the resistance to opsonization by SP-A. Interestingly, no significant differences were noted in SP-A-mediated phagocytosis when comparing 1244, PAO1, and PAK against their Δ*fgtA* mutants deficient in flagellin glycosylation (Fig. 6).

DISCUSSION

The pilin subunits of the group 1 *P. aeruginosa* strains, including strain 1244, are glycosylated with O-ag containing pseudaminic acid by the TfpO glycosyltransferase. TfpO of group I *P. aeruginosa* strains has been shown to be important for the overall fitness of 1244 in the host. In a mixed infection within mouse lungs, the Δ*tfpO* mutant is less competitive against its parental strain 1244 (11). However, the mechanism of attenuation is not clear. We proposed that one of the mechanisms of higher fitness in 1244 was due to the increased resistance to SP-A-mediated effects, afforded by the *O*-glycosylation of O-ag subunits to each pilin monomer on Tfp. Using a mouse model of acute pneumonia infection, we showed that 1244 caused mortality when inoculated into SP-A$^{-/-}$ mice but no mortality when inoculated in SP-A$^{+/+}$ mice. This demonstrates the importance of SP-A in innate immune resistance against 1244. Importantly, the cognate glycosyltransferase Δ*tfpO* mutant was attenuated during the infection of SP-A$^{+/+}$ mice, suggesting that O-ag contributes to the virulence of *P.*

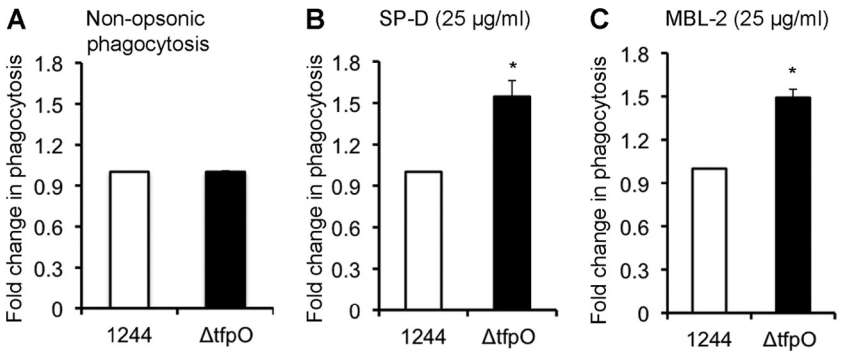

FIG 5 *O*-glycosylation of TFP with O-ag confers resistance to pulmonary and circulatory opsonins. (A) Phagocytosis of 1244 and the Δ*tfpO* mutant in the absence of opsonin. The changes in phagocytosis were normalized to strain 1244, which was set to the baseline value of 1. All phagocytosis experiments were independently performed three times in triplicates. The means ± the standard errors from a representative experiment are shown. (B and C) Phagocytosis of strain 1244 and the Δ*tfpO* mutant in the presence or absence of hSP-D and MBL-2. The ratio of ingested bacteria between animals exposed to hSP-D or MBL-2 versus those unexposed is expressed as the fold increase in phagocytosis. The changes in phagocytosis were then normalized to strain 1244, which was set to the baseline value of 1. All phagocytosis experiments were independently performed three times in triplicates. The means ± the standard errors from a representative experiment are shown. *, $P < 0.05$ when comparing the number of the phagocytosed Δ*tfpO* mutant against strain 1244 by using a parametric Student *t* test.

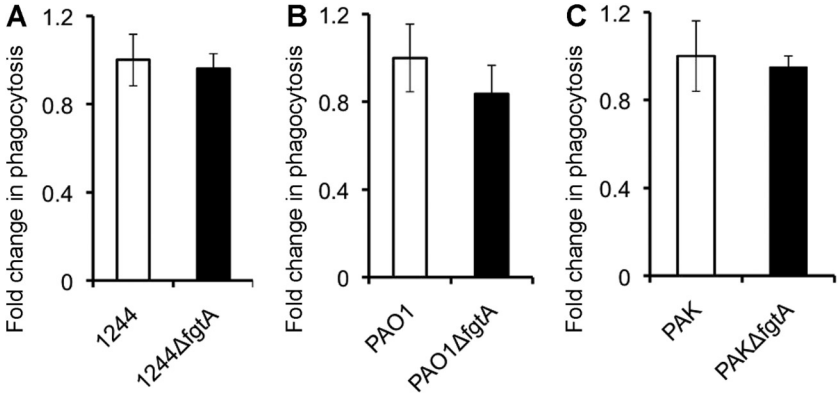

FIG 6 Glycosylation of flagella does not modulate resistance to SP-A-mediated opsonization. (A to C) RAW 264.7 macrophages were infected with 1244, PAO1, and PAK and their respective glycosylation-deficient Δ*rfbC* mutants in the presence or absence of hSP-A. The ratio of ingested bacteria between those exposed to hSP-A and those unexposed is expressed as the fold increase in phagocytosis. The changes in phagocytosis were then normalized to parental wild-type strains 1244, PAO1, and PAK, respectively, which were set to a baseline value of 1. All phagocytosis experiments were independently performed three times in triplicates. The means ± the standard errors from a representative experiment are shown. $P = 0.56$, 0.56, and 0.67 when comparing the number of phagocytosed 1244, PAO1, and PAK organisms to their respective Δ*tfpO* mutants by using a parametric Student *t* test.

aeruginosa strain 1244 against wild-type mouse lung expressing SP-A. This conclusion is supported by the finding that the Δ*tfpO* mutant burden is significantly higher in the SP-A$^{-/-}$ mice than in the SP-A$^{+/+}$ mice. Furthermore, the Δ*tfpO* mutant was able to cause moderate bronchopneumonia in the SP-A$^{-/-}$ mice but was only able to cause mild bronchopneumonia in the SP-A$^{+/+}$ mice. These results suggest that *O*-glycosylation of Tfp with O-ag confers resistance to antimicrobial properties of SP-A.

To further decipher the mechanism of SP-A resistance by 1244, we examined whether the O-ag on the pilin subunits of Tfp confers resistance to SP-A-mediated opsonization or membrane permeability. The presence of O-ag on pilin subunits of Tfp does not increase the resistance to SP-A-mediated membrane permeability. Instead, we show that *O*-glycosylation of Tfp with O-ag confers resistance to SP-A-mediated opsonization and clearance through phagocytosis. Under both *in vitro* and *in vivo* experimental conditions, the *O*-glycosylation-deficient Δ*tfpO* mutant was more susceptible to opsonization by hSP-A. However, the increase in the phagocytosis of the Δ*tfpO* mutant is most prominent *in vivo*. This is not surprising because the Δ*tfpO* mutant is also susceptible to opsonization by additional pulmonary and circulatory opsonins present within the alveolar surfactant layer, including SP-D and MBL-2.

Previous studies in other microbes have implicated the importance of O-ag for resistance to phagocytosis, including *Vibrio anguillarum* (47), *Burkholderia cenocepacia* (48), *Salmonella enterica* (49), *Escherichia coli* (50, 51), *Haemophilus ducreyi* (52), and *Neisseria gonorrhoeae* (53). In *E. coli*, *S. enterica*, *H. ducreyi*, and *N. gonorrhoeae*, it has been noted that O-ag confers resistance to phagocytosis by masking the *N*-acetylglucosamine residues of LPS core polysaccharide, which is the ligand for the dendritic cell-specific intercellular adhesion molecule nonintegrin (DC-SIGN)/CD209 (51, 52). However, the role of Tfp glycosylation with O-ag has not been studied with regard to resistance to SP-A or other opsonins. The results of the present study indicate that *O*-glycosylation of Tfp confers not only resistance to phagocytosis by pulmonary opsonins SP-A and SP-D but also resistance to circulatory opsonin MBL-2, which presents in lesser abundance in the lung.

Macrophage mannose receptor, a pattern recognition receptor for various microorganisms, has been shown to be important for the phagocytosis of microbes (53). In *V. anguillarum*, it is speculated that O-ag masks putative mannose residues that interact with the mannose receptors of skin epithelial cells (47). SP-A has been shown to upregulate the expression of mannose receptor (54). Upregulation of mannose receptor by SP-A and SP-D is important in the phagocytosis of *Mycobacterium avium* by monocyte-derived macrophages (55). Our preliminary analyses suggest that SP-A-upregulated mannose receptor plays a minimal role in enhancing the phagocytosis of the Δ*tfpO* mutant. This is because mannose at concentrations ≥1 mM or ~25 times higher than physiological levels (56) only minimally attenuates phagocytosis of Δ*tfpO* bacteria in the RAW 264.7 murine macrophages (R. M. Tan and G. W. Lau, unpublished data). However, we cannot rule out the possibility that this discrepancy may be due to different macrophages used in the studies. We are currently examining the interactions between Tfp glycosylation, SP-A, and mannose receptor using monocyte-derived macrophages.

In summary, we have shown that the glycosylation of O-ag subunit to Tfp enhances the virulence of *P. aeruginosa* during acute pneumonia infection. The increase in virulence is associated with the resistance to both pulmonary opsonins SP-A and SP-D, as well as the circulatory opsonin MBL-2, which is found at low concentrations in the lung. Given that antibiotic-resistant *P. aeruginosa* is a serious clinical issue, there is an added urgency to explore the use of novel, non-antibiotic-based antibacterial peptides to combat life-threatening infections. Unraveling the mechanisms governing resistance or susceptibility to SP-A may potentially lead to new treatment strategies for life-threatening lung infections. Future efforts will investigate the feasibility of using drugs to disrupt Tfp glycosylation, which may augment the clearance of *P. aeruginosa* in an opsonin-dependent manner, without increasing the burden of antibiotic resistance.

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