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# Type III Secretion-Dependent Modulation of Innate Immunity as One of Multiple Factors Regulated by *Pseudomonas aeruginosa* RetS

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**Mutation of *retS* (*rtsM*) of *Pseudomonas aeruginosa* strain PA103 reduces its virulence in both ocular and respiratory murine models of infection. In vitro, *retS* mutants exhibit loss of the ExsA-regulated type III secretion system (TTSS), reduced twitching motility, and a decrease in association with, invasion of, and survival within corneal epithelial cells. In addition, transcription of multiple other virulence genes is positively and negatively affected by *retS* mutation. Since our published data show that ExoU and ExoT, the two TTSS effectors encoded by strain PA103, each confer virulence in this corneal model, we hypothesized that loss of virulence of *retS* mutants follows loss of type III secretion. Corneal pathology, bacterial colonization, and phagocyte infiltration were compared for wild-type PA103, *retS* mutants, and various TTSS mutants after infection with ~$10^6$ CFU bacteria. Results showed that either a *retS* or an *exsA* (TTSS) mutation delayed disease progression, as illustrated by reduced severity scores and colonization levels during the first 48 h postinfection. Surprisingly, *retS* mutant infections then became more severe than those involving *exsA* mutants. By day 7, colonization levels of *retS* mutants even surpassed those of wild-type bacteria (more than twofold, $P = 0.028$). Although *retS* mutants caused more severe opacification of central corneas than both the wild type and the *exsA* mutants, neither mutant caused the peripheral ring opacity commonly associated with wild-type infection, suggesting that the TTSS was involved. Histological experiments with *retS* and various TTSS mutants showed that ring opacification required ExoU but not ExoT and that it consisted of dense polymorphonuclear phagocyte infiltration at the corneal periphery and the absence of any cell type in the central cornea. These data suggest that these *P. aeruginosa* TTSS effectors have different effects on innate immunity and that RetS influences virulence beyond its effects on the TTSS.**

Recently, we reported that *retS*, a gene encoding a novel *Pseudomonas aeruginosa* hybrid sensor-response regulator protein, is involved in corneal virulence in vivo during the first 24 h of the disease (42). In vitro analysis revealed that mutation of *retS* in strain PA103 caused loss of type III secretion and a reduction in multiple epithelium-associated virulence mechanisms, including; twitching motility, epithelial association, invasion, and intracellular survival (42). Two other recent publications by others show that RetS (regulator of exopolysaccharide and type III secretion), also designated RtsM (regulator of type III secretion), participates in virulence during acute pneumonia (16 to 18 h postinoculation) (20, 27). These studies also showed loss of the ExsA-regulated type III secretion system (TTSS) in *retS* and *rtsM* mutants in vitro. This could be restored by overexpression of ExsA (27). One of these studies also showed that RetS positively and negatively affects the expression of many genes in addition to regulation of type III secretion (20). For example, *retS* mutation caused downregulation of genes involved in type IV pilus expression and type II secretion and upregulation of the *psl* and *pel* operon genes that are involved in exopolysaccharide production and biofilm formation (20).

Type III secretion contributes to the pathogenesis of septic shock and the virulence of *P. aeruginosa* in the cornea (28, 29), burn wounds (24, 25), and the airways (1, 2, 11, 26, 30, 32, 33, 36, 39). Four known effector proteins are delivered into mammalian cells by this TTSS: ExoS, ExoT, ExoU, and ExoY. Each effector can significantly alter mammalian cell function in vitro. For example, ExoS and ExoT each have both ADP-ribosyltransferase (C-terminal) and RhoGAP (N-terminal) activities, ExoU is a potent phospholipase, and ExoY is an adenylate cyclase (3, 35). Although each effector has been shown to affect cellular health in vitro, e.g., ExoU is cytotoxic to epithelial cells and phagocytes (12, 21) and ExoT is antiphagocytic and inhibits epithelial wound healing (7, 17–19), the contributions of these in vitro biological activities to disease pathogenesis in vivo are not fully understood.

Of the known TTSS effectors of *P. aeruginosa*, strain PA103 expresses only ExoU and ExoT; it does not encode either ExoS or ExoY. Using strain PA103 in a murine model, we previously showed that ExoU and ExoT were each sufficient, when exposed alone, to promote corneal colonization and pathology (28). Double mutation of both *exoU* and *exoT* was required to reduce corneal disease severity, coinciding with a >1,000-fold decrease in bacterial tissue colonization. That finding shows requirement redundancy between ExoU and ExoT, yet these effectors have no known common activities. In animal models of acute pneumonia, double mutation of *exoU* and *exoT* attenuates lung injury (11). ExoU has been found to be critical for causing local disease pathology (2, 12, 36), with ExoT contributing to disease by enhancing the systemic spread of bacteria (17).

Since the TTSS is involved in corneal disease, it is likely that

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TABLE 1. *P. aeruginosa* strains and mutants used in this study

| Strain | Relevant characteristics | Reference(s) |
|---|---|---|
| Wild-type PA103 | Functional ExsA-regulated TTSS; expresses two known effectors, ExoU and ExoT | 13 |
| Mutant PA103*retS*::Tn*5* | Tn*5* insertion in *retS*, loss of ExsA-regulated TTSS and other epithelium-associated virulence mechanisms, upregulation of other virulence mechanisms, e.g., biofilm formation and exopolysaccharide expression | 20, 27, 42 |
| Mutant PA103*exsA*::Ω | Ω insertion in *exsA*, loss of ExsA-regulated TTSS and unknown ExsA-dependent virulence mechanisms | 16 |
| Mutant PA103*exoU* Tn*5*::Tc$^r$ | Tn*5* insertion in *exoU*, loss of ExoU, expression of functional ExsA-regulated TTSS and effector ExoT | 12 |
| Mutant PA103*exoT*::Tc$^r$ | Tn insertion in *exoT*, loss of ExoT, expression of functional ExsA-regulated TTSS and effector ExoU | 12 |
| Mutant PA103*exoU exoT*::Tc$^r$ | Expression of functional ExsA-regulated TTSS, no expression of ExoU or ExoT | 38 |

at least part of the attenuated virulence of *retS* mutants during the first 24 h of the disease process involves loss of type III secretion. However, other virulence mechanisms regulated by *retS* are also thought to contribute to *P. aeruginosa* infection and disease pathology. These include type II secretion and type IV pili, the latter contributing to *P. aeruginosa* virulence in vivo via their role in surface-associated twitching motility and likely through their contributions to epithelial association, invasion, and cytotoxicity (6, 14, 15, 37, 41, 43).

In this study, we explored the relationship between reduced virulence of *retS* mutants of *P. aeruginosa* and loss of the TTSS. We hypothesized that loss of the TTSS would account for the reduced virulence of *P. aeruginosa retS* mutants. To test this hypothesis, we compared *retS* mutant infections to disease caused by wild-type *P. aeruginosa* strain PA103 and that caused by an isogenic *exsA* (transcriptional activator of the TTSS), *exoU*, *exoT*, or *exoU exoT* mutant by using the 6-h healing model of corneal infection (29). Infected eyes were examined for disease severity and bacterial colonization and by histological methods during the first 7 days postinfection.

## MATERIALS AND METHODS

**Bacterial strains.** The *P. aeruginosa* strains and mutants used in this study are presented in Table 1. Bacterial inocula were prepared from overnight cultures grown on Trypticase soy agar plates at 37°C before suspension in minimal essential Eagle medium with Hanks' salts and L-glutamine (Sigma, St. Louis, MO) buffered with 1 M HEPES-NaOH (pH 7.6) and 0.35 g of $NaHCO_3$ and 6 g of bovine serum albumin (Sigma, St. Louis, MO) per liter (MEM). The bacteria were initially prepared to a concentration of $\sim 1 \times 10^9$ CFU/ml of MEM as determined by spectrophotometry (optical density at 650 nm of ~1.0). Bacterial numbers were confirmed by viable counts after serial dilution.

**Corneal infection model.** The 6-h healing murine model of corneal infection was used since we had previously used it to demonstrate the role of ExsA in *P. aeruginosa* corneal penetration in vivo and the contribution of RetS to early (<24 h) corneal virulence (29, 42). Following anesthesia, three full-thickness epithelial abrasions were produced on the left corneas of 6-week old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) with a sterile 26-guage needle. After a 6-h healing period, the corneas were inoculated with 5 μl of a bacterial suspension (containing between $\sim 10^6$ and $\sim 10^7$ CFU of bacteria). Three mice were assigned to each sample group for pilot experiments; five mice per group were used for experiment repeats. Disease was assessed at 1, 2, 4, and 7 days postinoculation by a masked investigator with a stereomicroscope, and corneal pathology was photographed. The following grading scheme was used (5): grade 0, eye macroscopically identical to the uninfected contralateral control eye; grade 1, faint opacity partially covering the pupil; grade 2, dense opacity covering the pupil; grade 3, dense opacity covering the entire anterior segment; grade 4, perforation of cornea and/or phthisis bulbi (shrinkage of the eyeball following inflammatory disease). All procedures were conducted in accordance with the policies established by the Association for Research in Vision and Ophthalmology and were approved by the University of California, Berkeley, Animal Care and Use Committee.

**Bacterial colonization.** To assess bacterial ocular colonization, mice were euthanatized and infected eyes were then immediately enucleated and homogenized in tryptic soy broth (1 ml). Viable counts were then performed on the homogenates.

**Histological examination of infected corneas.** Eyes were fixed in 4% paraformaldehyde and embedded in medium-grade LR white, and then 1-μm sections were cut with an ultramicrotome (Leica Ultracut R). These were stained with toluidine blue and observed with an Olympus IX-70 inverted microscope. Representative sections were photographed and analyzed with the Improvision computer analysis system, which was also used to quantify polymorphonuclear phagocyte (PMN) numbers and corneal thickness in the same sections of infected corneas.

**Statistical analysis.** Bacterial colonization data were expressed as a median with lower and upper quartiles. Differences between these groups were compared for statistical significance by a nonparametric test, i.e., the Mann-Whitney test (two groups) or the Kruskal-Wallis test (three or more groups). Other data were expressed as a mean with a standard deviation, and differences between groups were compared for statistical significance by Student's *t* test and analysis of variance.

## RESULTS

***P. aeruginosa retS* and *exsA* mutant infections showed delayed development compared to wild-type infections.** Corneal infections caused by wild-type PA103 were compared to those caused by *retS* and *exsA* (type III secretion) mutants at days 1, 2, 4, and 7 postinfection by using photographs of infected corneas (Fig. 1A and B) and disease severity scores (Table 2). Infections with either of the mutants were less severe than wild-type infections at days 1, 2, and 4 ($P < 0.05$, Mann-Whitney test, comparison of *retS* and *exsA* mutants versus wild-type PA103 at each time point) but by day 7 were of similar overall severity to those caused by wild-type bacteria ($P > 0.05$, Mann-Whitney test) (Fig. 1B; Table 2). Between days 2 and 7, *retS*-induced corneal disease pathology developed more rapidly than that induced by the *exsA* mutant, as evidenced by a significant increase in disease severity scores at day 4 ($P < 0.05$, Mann-Whitney test) (Fig. 1B; Table 2). Although the *retS* mutant did not differ from the wild type in overall disease severity scores (Table 2), it was evident that *retS* mutants caused more severe opacification of the central cornea than the wild type at day 7 (Fig. 1B).

***P. aeruginosa retS* and *exsA* mutants showed reduced ocular colonization compared to the wild type at 48 h but not at 7 days.** Bacterial viable counts were used to compare colonization of infected eyes at 4 h, 48 h, and 7 days postinoculation

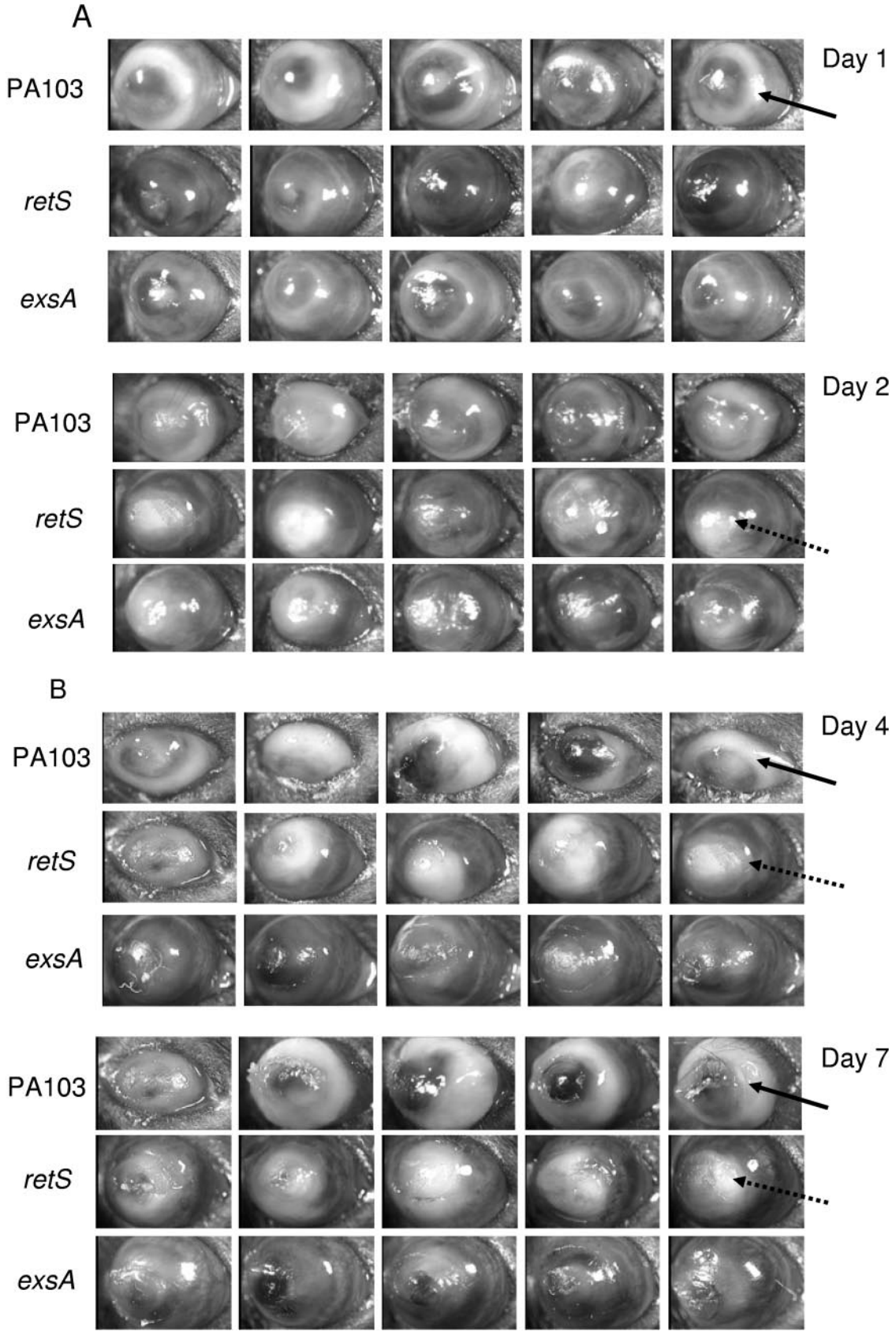


FIG. 1. Corneal infections caused by *P. aeruginosa* wild-type strain PA103 compared to those caused by *retS* (PA103*retS*::Tn*5*) and *exsA* (PA103*exsA*::Ω) mutants at days 1 and 2 (A) and 4 and 7 (B) postinfection with $\sim 10^6$ CFU of bacteria in the 6-h healing murine model of infection. Solid arrows indicate peripheral ring infiltrates, and dashed arrows show dense central infiltrates. Within each group, the same mice were followed throughout the course of infection.

TABLE 2. Ocular disease severity scores of mouse corneas over 7 days postinoculation with *P. aeruginosa* strain PA103 or the *retS* or *exsA* mutant in the 6-h healing model of corneal infection (29)

| Strain | Infecting dose (no. of CFU [$10^6$] in 5 μl) | Ocular scores[a] | | | |
|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 4[c] | Day 7 |
| PA103[b] | 4.2 | 2.5, 3, 3, 3, 3 | 3, 4, 4, 4, 4 | 4, 4, 4, 4, 4 | 4, 4, 4, 4, 4 |
| *retS* mutant | 2.9 | 1, 1, 2, 2.5, 3 | 2, 2.5, 3, 3, 3 | 3, 3, 3.5, 3.5, 4 | 4, 4, 4, 4, 4 |
| *exsA* mutant | 3.0 | 2, 2, 2, 2, 2.5 | 2, 2.5, 2.5, 3, 3 | 2, 3, 3, 3, 3 | 3, 3, 3.5, 4, 4 |

[a] Defined in Materials and Methods.
[b] The increased disease severity due to PA103 over that due to both the *retS* and *exsA* mutants was significant on days 1, 2, and 4 ($P < 0.05$, Mann-Whitney test) but not on day 7.
[c] The increased disease severity due to the *retS* over that due to the *exsA* mutant after 4 days was significant ($P < 0.05$, Mann-Whitney test).

TABLE 4. Ocular colonization by *P. aeruginosa* strain PA103 and the *retS* and *exsA* mutants at 48 h postinoculation in the 6-h healing murine model of corneal infection

| Strain | Infecting dose (no. of CFU [$10^6$] in 5 μl) | Median final viable count (CFU/ml) (lower quartile–upper quartile)[a] at 48 h |
|---|---|---|
| PA103 | 1.9 | $2.1 \times 10^7$ ($1.6 \times 10^7$–$2.3 \times 10^7$) |
| *retS* mutant | 2.6 | $7.8 \times 10^5$ ($5.5 \times 10^5$–$3.1 \times 10^6$)[b] |
| *exsA* mutant | 1.8 | $2.1 \times 10^5$ ($1.9 \times 10^5$–$5.2 \times 10^5$) |

[a] Significant difference in colonization among all groups ($P = 0.004$, Kruskal-Wallis test).
[b] Increased colonization (3.7-fold) by the *retS* mutant over that by the *exsA* mutant ($P < 0.05$, Mann-Whitney test).

with wild-type bacteria and each of the mutants (Tables 3, 4, and 5). No significant differences were found in early (4 h) colonization between eyes infected with PA103 or the *retS* or *exsA* mutant (Table 3). By 2 days postinfection, however, both the *retS* and *exsA* mutants showed significantly reduced ocular colonization compared to the wild type (~27-fold reduction for the *retS* mutant; ~100-fold reduction for the *exsA* mutant) (Table 4). The 3.7-fold colonization advantage shown by the *retS* mutant over the *exsA* mutant at 48 h was statistically significant ($P < 0.05$, Mann-Whitney test) (Table 4). This colonization advantage continued later in the disease; indeed, at 7 days, the *retS* mutant showed greater ocular colonization (more than twofold) than either *exsA* mutant or wild-type bacteria ($P < 0.05$, Mann-Whitney test in each case) (Table 5). Ocular colonization by PA103 and that by the *exsA* mutant did not differ at 7 days ($P = 0.754$, Mann-Whitney test).

***P. aeruginosa retS* and *exsA* mutants differ from the wild type in PMN infiltrative responses.** Wild-type *P. aeruginosa* caused peripheral ring infiltration (solid arrows) at all time points (Fig. 1A and B). In contrast, *retS* and *exsA* mutant infections were each associated with dense infiltrates of the central cornea (dashed arrows, Fig. 1A and B). Histological examination of wild-type-infected eyes at 48 h postinoculation indicated an absence of PMN infiltration of the central cornea with dense clusters of PMNs localized at the corneal periphery (Fig. 2A, top). In contrast, in eyes infected with the *retS* or *exsA* mutant (Fig. 2A, middle and bottom, respectively), PMNs infiltrated both the central and peripheral regions of the cornea. PMN distribution gradients observed histologically correlated with peripheral ring and dense central infiltration pathology observed macroscopically for wild-type versus mutant bacterial infections, respectively (Fig. 2B). These results showed that ring infiltration involved repression of PMN infiltration of the central cornea and was a property of the TTSS. Subsequently, the role of the TTSS effectors expressed by PA103 (ExoU and ExoT) in this effect was explored.

**ExoU is required for repression of PMN infiltration of the central cornea.** Corneal infections involving wild-type *P. aeruginosa* (PA103) or the *retS* mutant were compared with those caused by the *exoU*, *exoT*, and *exoU exoT* mutants at 48 h to determine the relative contributions of ExoU and ExoT to repression of PMN infiltration and the relationship to bacterial colonization. Double mutation of *exoU* and *exoT* caused loss of ring infiltration, similar to *retS* and *exsA* mutants, showing that one or both of these effectors was required (Fig. 3, dashed arrows). Experiments with mutants with only one mutated effector showed that ExoU was required for ring infiltration since the *exoT* mutant (still expresses ExoU) caused ring infiltrates (Fig. 3, solid arrows) while the *exoU* mutant did not. Histological analysis (Fig. 4) confirmed these macroscopic observations by showing localization of PMNs in the corneal periphery for the *exoT* mutant (Fig. 4C, left) similar to the wild type (Fig. 4A, left) with the central corneas devoid of PMNs but loaded with bacteria (Fig. 4A and C, right). In contrast, central and peripheral corneas each contained large numbers of PMNs when infected with the *exoU* mutant (Fig. 4B), similar to those infected with the *exoU exoT* double mutant (Fig. 4D).

PMN distribution within infected murine corneas at 48 h postinoculation with wild-type *P. aeruginosa* (strain PA103) or the *exoU*, *exoT*, or *exoU exoT* mutant was quantified (Fig. 5). Results confirmed the histological observations. There were PMN concentration gradients among the peripheral, precentral, and central regions of the infected corneas. Significantly more PMNs per square micrometer of tissue were counted in the peripheral versus the precentral and central regions of corneas infected with wild-type *P. aeruginosa* or the *exoT* mu-

TABLE 3. Ocular colonization by *P. aeruginosa* strain PA103 and the *retS* and *exsA* mutants at 4 h postinoculation in the 6-h healing murine model of corneal infection

| Strain | Infecting dose (no. of CFU [$10^6$] in 5 μl) | Median final viable count[a] (CFU/ml) (lower quartile–upper quartile) at 4 h |
|---|---|---|
| PA103 | 2.5 | $6.5 \times 10^4$ ($2.5 \times 10^4$–$1.5 \times 10^5$) |
| *retS* mutant | 2.9 | $1.1 \times 10^5$ ($9.5 \times 10^4$–$3.9 \times 10^5$) |
| *exsA* mutant | 2.1 | $6.5 \times 10^4$ ($2.5 \times 10^4$–$1.3 \times 10^5$) |

[a] No significant difference in colonization among all groups ($P = 0.276$, Kruskal-Wallis test).

TABLE 5. Ocular colonization by *P. aeruginosa* strain PA103 and the *retS* and *exsA* mutants at 7 days postinoculation in the 6-h healing murine model of corneal infection

| Strain | Infecting dose (no. of CFU [$10^6$] in 5 μl) | Median final viable count (CFU/ml) (lower quartile–upper quartile) at 7 days |
|---|---|---|
| PA103 | 4.2 | $6.1 \times 10^6$ ($5.2 \times 10^6$–$7.0 \times 10^6$) |
| *retS* mutant | 2.9 | $1.3 \times 10^7$ ($9.2 \times 10^6$–$1.4 \times 10^7$)[a] |
| *exsA* mutant | 3.0 | $5.8 \times 10^6$ ($5.4 \times 10^6$–$6.3 \times 10^6$) |

[a] A more-than-twofold increase in colonization by *retS* over PA103 or the *exsA* mutant ($P < 0.05$ [Mann-Whitney test] for each case).

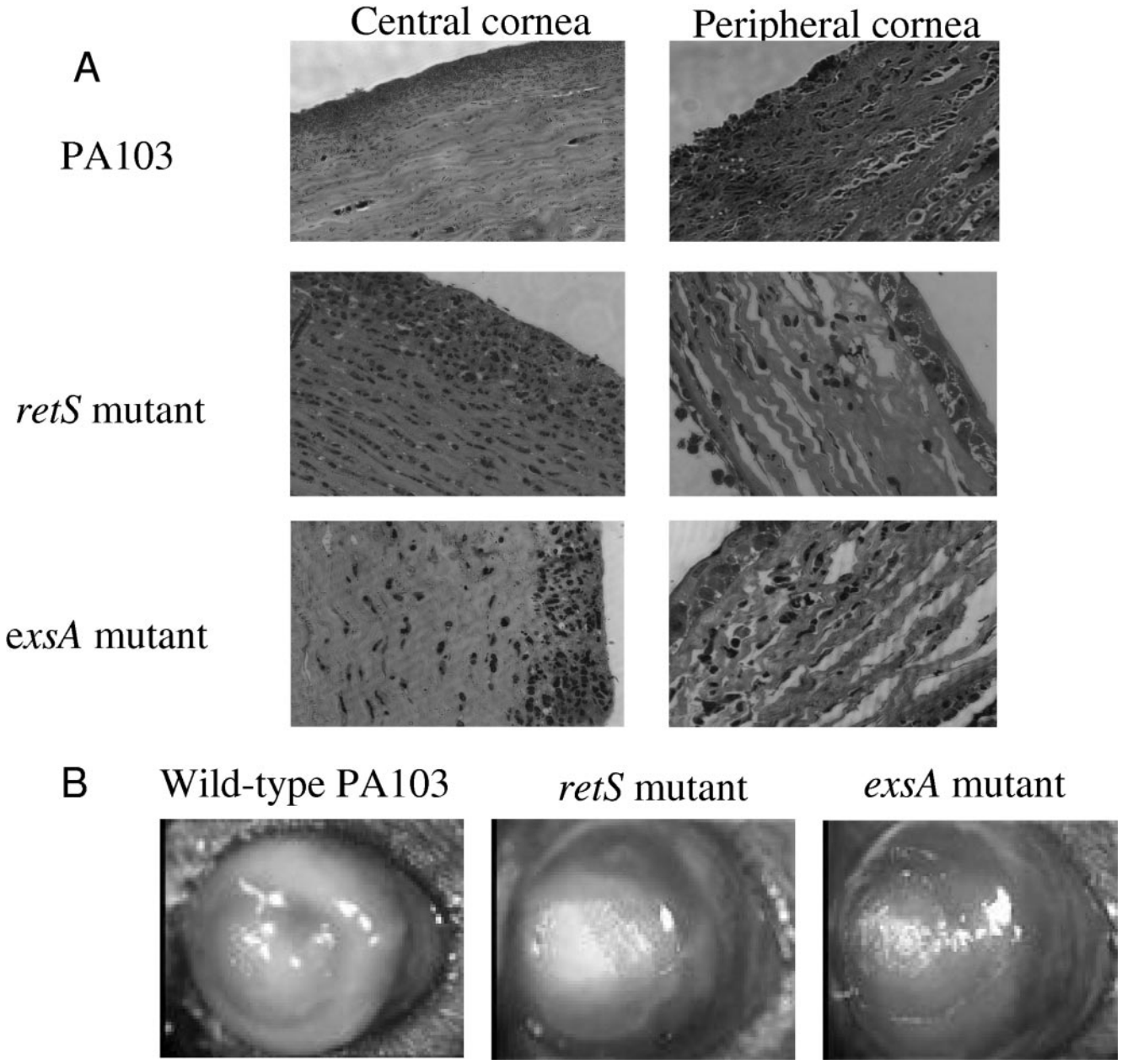


FIG. 2. Histology (A) and pathology (B) of corneal disease at 2 days (48 h) postinoculation with *P. aeruginosa* strain PA103 or the *retS* or *exsA* mutant at $\sim 10^6$ CFU/5 μl in the 6-h healing murine model of infection. PMN infiltration of the central cornea was observed in eyes infected with the *retS* and *exsA* mutants. However, PMN migration appeared restricted to the peripheral cornea in eyes infected with wild-type PA103 (×1,200 magnification [A]).

tant. The inverse relationship existed in corneas infected with the *exoU* mutant and the *exoU exoT* double mutant. Differences in PMN distribution correlated with differences in corneal thickness, a measure of the overall inflammatory response, for wild-type bacteria and the *exoU exoT* double mutant but not for the *exoU* or *exoT* mutant (Fig. 6).

The colonization results of these experiments (Table 6) confirmed our previously published data (28). There were significant reductions in ocular colonization at 48 h for the *exoU exoT* double mutant (28) compared to both wild-type bacteria and *retS* mutant bacteria ($P < 0.05$, Mann-Whitney test) (Table 6). Mutation of either *exoU* or *exoT* did not cause a loss of tissue colonization (Table 6), despite PMN migration into the central cornea when the eye was colonized by the *exoU* mutant (still expresses ExoT).

## DISCUSSION

The results of this study showed that the attenuation of *P. aeruginosa* virulence by the *retS* mutation is temporary, only lasting for the first few days of the disease. By day 7 postinfection, colonization rates by the *retS* mutant exceeded those of

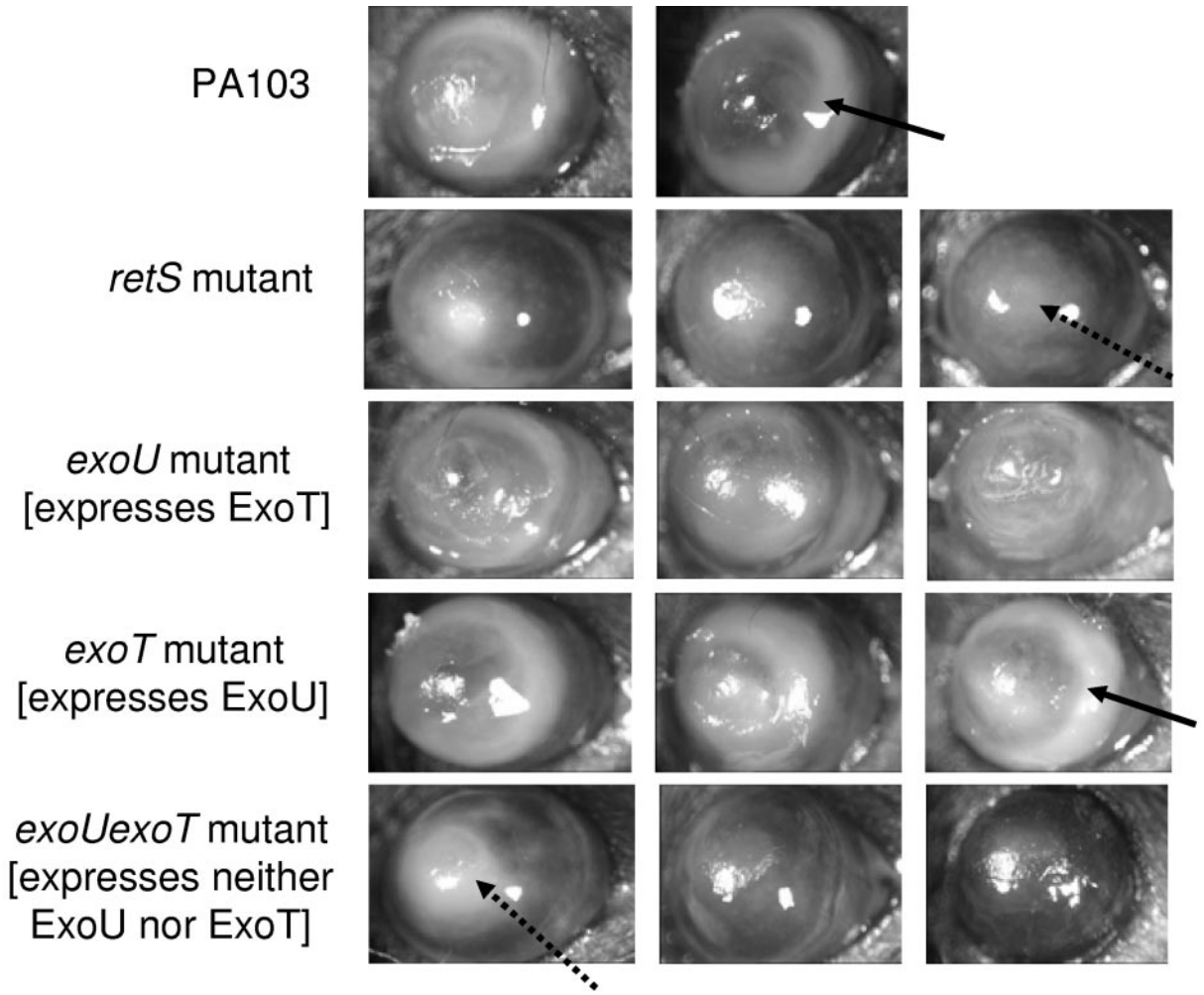


FIG. 3. Corneal pathology at 2 days (48 h) postinoculation with *P. aeruginosa* strain PA103 or the *retS*, *exoU*, *exoT*, or *exoU exoT* mutant at ~$10^6$ CFU/5 μl in the 6-h healing murine model of infection. Peripheral ring infiltrates (solid arrows) were observed with wild-type bacteria and the *exoT* mutant (still expresses ExoU). In contrast, pathology observed with the *retS* and *exoU exoT* mutants predominantly involved infiltrates in the central cornea (dashed arrows). The *exoU* mutant (expresses ExoT) caused pathology involving both the central and peripheral regions of the cornea.

wild-type bacteria. Similarities between *retS* and TTSS mutants included lack of peripheral ring opacity, which in wild-type-infected eyes was found to consist of dense peripheral PMN infiltrates with a corresponding absence of cells at the central cornea. Peripheral ring opacity was shown to require ExoU, one of two type III secreted effectors encoded by *P. aeruginosa* strain PA103. Comparison of TTSS and *retS* mutants later in the disease process (48 h through 7 days) revealed significant increases in both colonization rates and disease severity scores in favor of the *retS* mutants showing that *retS* mutation influences virulence through mechanisms additional to loss of the TTSS.

We, and others, have previously reported attenuation of virulence during the first 24 h postinfection with *retS* mutant *P. aeruginosa* (20, 27, 42). In the present study, we found attenuation of virulence for the *retS* mutant at 48 h and 4 days. Since TTSS mutants were also attenuated at these time points, it is likely that the defect in TTSS contributes to virulence loss for the *retS* mutant. However, virulence loss could also involve downregulation of genes involved in type IV pilus expression, type II secretion, and/or a reduced ability to associate with, invade, and survive within epithelial cells (20, 41–43).

RetS has been shown to negatively regulate the expression of various genes. Therefore, it is interesting that the *retS* mutant caused more severe disease (central corneal opacification) and colonized more efficiently than both TTSS mutant and wild-type bacteria at later time points. Genomic transcriptional profiling suggested that *retS* mutation switched the *P. aeruginosa* phenotype from one suited to acute infection to one more suited to chronic colonization and disease (20). For example, both *psl* and *pel* operons encoding exopolysaccharide expression and promoting biofilm formation are upregulated in the *retS* mutant (20). This could compensate for loss of TTSS by directly contributing to increased bacterial colonization and corneal disease pathology or indirectly if it were to inhibit bacterial clearance by professional phagocytes (31).

Neither the *retS* mutant nor the *exsA* mutant caused peripheral ring opacity during disease, which required the TTSS effector ExoU and involved repression of PMN infiltration of the central cornea. The ability of ExoU to affect innate im-

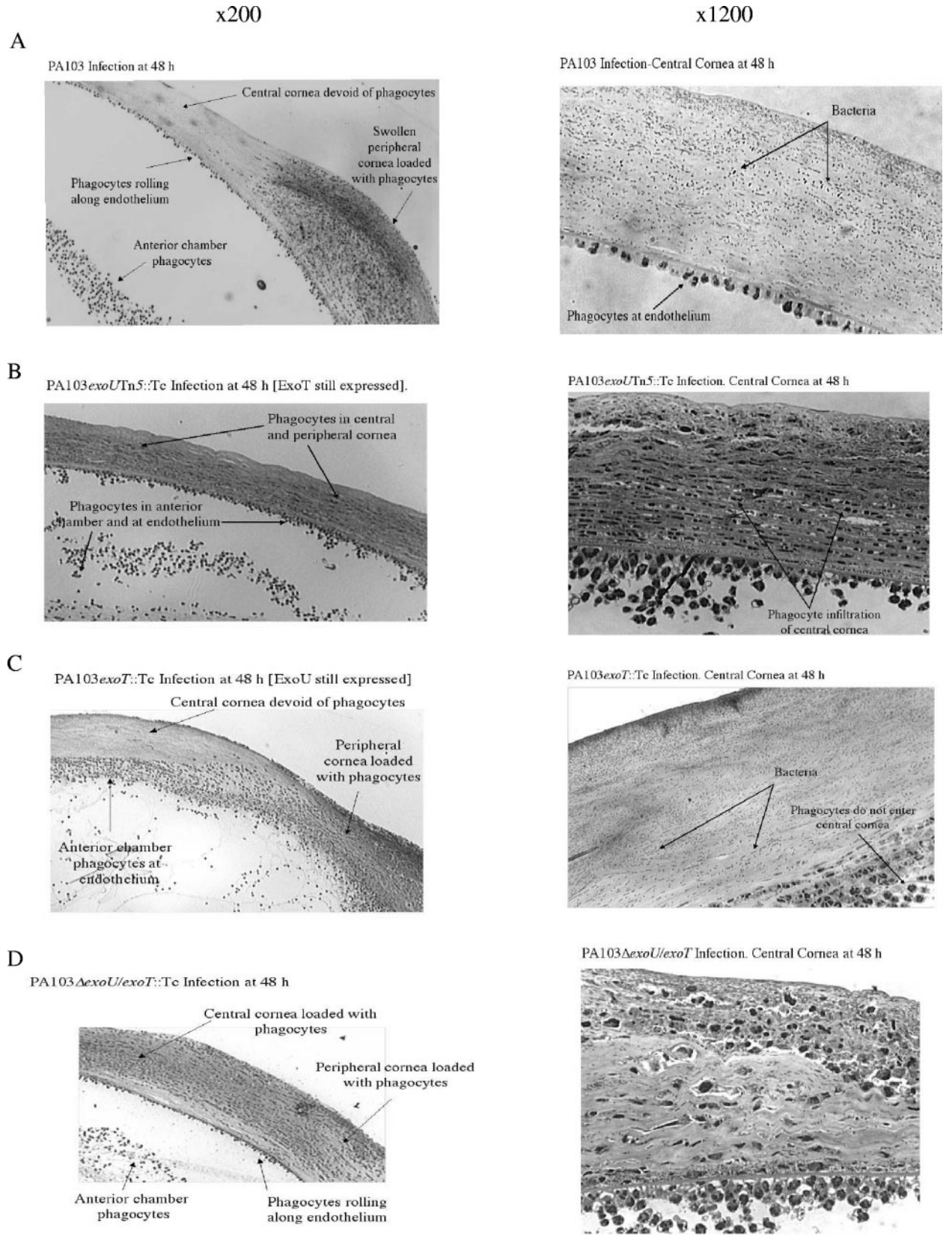


FIG. 4. Corneal histology at 2 days (48 h) postinoculation with *P. aeruginosa* strain PA103 or the *exoU*, *exoT*, or *exoU exoT* mutant at ~$10^6$ CFU/5 μl in the 6-h healing murine model of infection. Extensive PMN infiltration of the central cornea was observed in eyes infected with the *exoU* and *exoU exoT* mutants. However, PMN migration was restricted to the peripheral regions of the cornea in eyes infected with wild-type PA103 and the *exoT* mutant (×200 magnification, left side; ×1,200 magnification, right side).

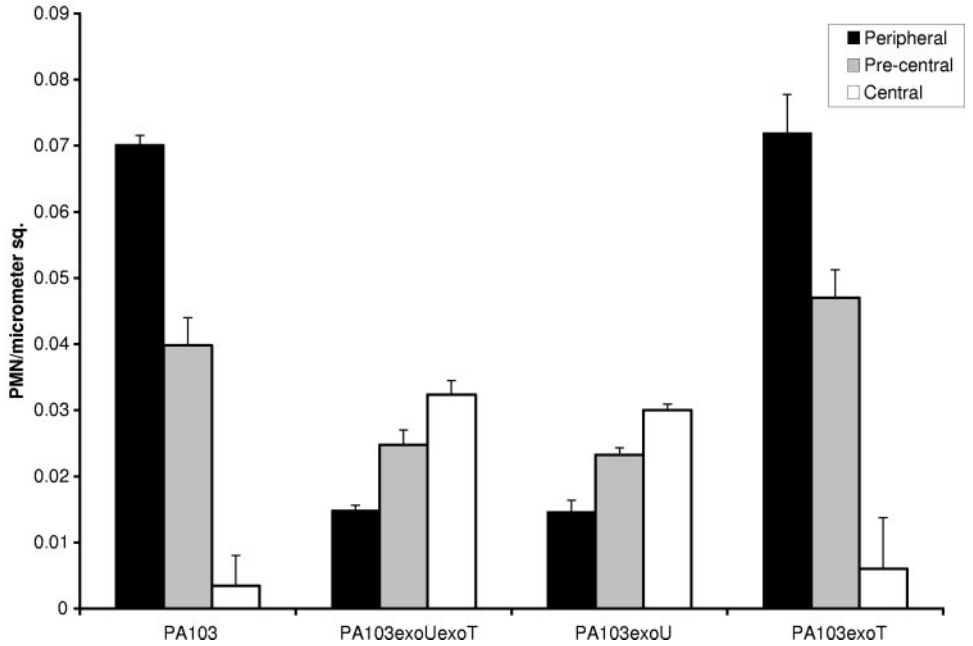


FIG. 5. Distribution of PMNs among the peripheral, precentral, and central regions of mouse corneas at 48 h postinoculation with *P. aeruginosa* strain PA103 or the *exoU*, *exoT*, or *exoU exoT* mutant.

mune responses in this fashion is likely to relate to the mechanism by which it promotes colonization in vivo (2, 28). PMN infiltration of the cornea in response to bacterial infection represents a key part of the ocular innate immune response to bacterial infection (22). During infection, these cells migrate into the cornea and anterior chamber from the limbus (junction of the peripheral cornea and conjunctiva), from the tear film, and/or from other vascularized ocular tissues, e.g., the uveal tract. Chemotactic cytokines, e.g., interleukin-1 and macrophage inflammatory protein 2 (murine equivalent of interleukin-8) play an important role in PMN recruitment (22, 34, 40). PMN infiltration assists in clearing bacteria but also contributes greatly to corneal pathology during infection (5, 22, 23, 34).

The mechanism by which ExoU inhibits phagocyte infiltration might involve direct killing of these infiltrating cells (21). Alternatively, it might follow ExoU-mediated killing of other cell types that normally reside in the central cornea and that would otherwise produce the chemotactic cytokines that recruit phagocytes into infected corneas (22, 34, 40). Indeed, corneas with ring opacities were found to be completely devoid of all types of resident corneal cells in the central region.

It was interesting that ExoT did not also suppress PMN infiltration, since our previous studies have shown ExoT to be almost as effective as ExoU in promoting bacterial colonization. It is most possible that ExoT promotes bacterial survival, despite infiltration of numerous PMNs, by inhibiting phagocytosis through its N-terminal RhoGAP activity (17), as has been shown in vitro. Alternatively, ExoT promotion of colonization could involve antiphagocytic effects on epithelial cells (7), since

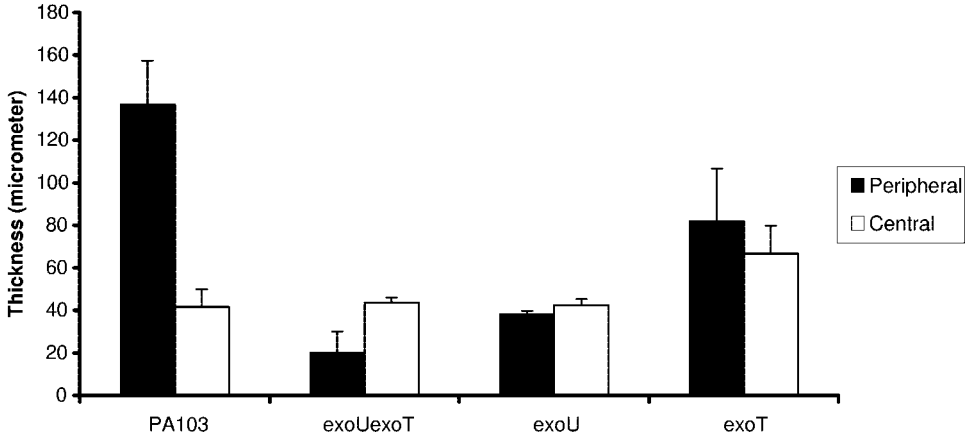


FIG. 6. Thickness of peripheral and central regions of murine corneas at 48 h postinfection with *P. aeruginosa* strain PA103 or the *exoU*, *exoT*, or *exoU exoT* mutant.

TABLE 6. Tissue colonization by wild-type PA103 and *retS*, *exoU*, *exoT*, and *exoU exoT* mutants in the 6-h healing murine model of corneal infection at 48 h postinoculation

| Strain | Infecting dose (no. of CFU [$10^6$] in 5 μl) | Median final viable count (CFU/ml) (lower quartile–upper quartile) |
|---|---|---|
| PA103 | 2.3 | $2.40 \times 10^7$ ($2.00 \times 10^7$–$2.85 \times 10^7$) |
| *retS* mutant | 3.3 | $2.55 \times 10^5$ ($1.55 \times 10^5$–$4.80 \times 10^5$)[a] |
| *exoU* mutant | 2.5 | $2.75 \times 10^7$ ($0.77 \times 10^7$–$3.90 \times 10^7$) |
| *exoT* mutant | 3.0 | $2.25 \times 10^7$ ($1.55 \times 10^7$–$2.70 \times 10^7$) |
| *exoUT* mutant | 3.1 | $1.65 \times 10^4$ ($0.79 \times 10^4$–$1.80 \times 10^4$) |

[a] A 15-fold increase in colonization by *retS* over that by the *exoU exoT* double mutant ($P < 0.05$, Mann-Whitney test).

sloughing of surface corneal epithelial cells containing bacteria that have invaded is thought to represent a defense against infection (15). Inhibition of epithelial wound healing by ExoT (19) could also contribute to *P. aeruginosa* persistence in the cornea by enhancing bacterial tissue binding (13) or by promoting epithelial penetration to the underlying stroma, which we have shown to involve an ExsA-regulated factor(s) (29).

TTSS effectors of some other pathogens, including *Pseudomonas syringae* and *Yersinia* and *Salmonella* spp., have been found to suppress cellular innate immune responses by interfering with pathogen recognition receptors, e.g., Toll-like receptors, and their associated signal transduction pathways, that upregulate chemokines, antimicrobial peptides, and other components of innate immunity in response to pathogen-associated molecular patterns (4, 10). It is not known whether any of the TTSS effectors of *P. aeruginosa* exert this type of activity. Interestingly, in rodent models of acute pneumonia, the *P. aeruginosa* TTSS can contribute to elevated proinflammatory cytokine levels in bronchoalveolar lavage fluid via TLR4 signaling and coinciding with reduced PMN numbers and increased bacterial colonization (1, 11). However, the relationship, if any, between increased TTSS-mediated cytokine expression and reduced PMN numbers in the airways has not yet been elucidated.

Modulation of innate immunity by ExoU of *P. aeruginosa* may not be specific to the cornea. During lung infection, an *exoU exoT* double mutant of PA103 has been reported to cause increased PMN numbers in bronchoalveolar lavage fluid of mice compared to the wild-type bacterium (1). Whether or not ExoU is involved has yet to be determined. However, a different study with a rat lung infection model reported that another *P. aeruginosa* strain that does not encode ExoU (but does encode ExoS and ExoT) also demonstrates TTSS-dependent modification of PMN infiltration (1). Thus, TTSS-encoded proteins other than ExoU likely contribute to reduced PMN numbers in the airways, perhaps via cytotoxicity against these and other phagocytes, as reported in vitro (8, 9, 21).

In conclusion, the data presented in this report suggest that RetS (RtsM) contributions to *P. aeruginosa* virulence in the cornea include regulation of ExsA-mediated repression of phagocyte infiltration via ExoU and most likely other contributions by the TTSS. However, they also show that other factors regulated by RetS (RtsM) actually dampen pathological effects at later stages of the disease process irrespective of competence for type III secretion. This was evidenced by enhanced colonization and virulence of a type III secretion-disabled *retS* mutant compared to both *exsA* mutants and type III secretion-competent wild-type bacteria during later (4 to 7 days postinfection) stages of disease. Whether this involves factors negatively regulated by *retS* or even more complicated cross talk between the host and pathogen has yet to be determined.

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