Pseudomonas aeruginosa Small Protease (PASP), a Keratitis Virulence Factor

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PURPOSE. The virulence contribution of *Pseudomonas aeruginosa* small protease (PASP) during experimental keratitis was studied by comparing a PASP-deficient mutant with its parent and rescue strains.

METHODS. The *pasP* gene of *P. aeruginosa* was replaced with the tetracycline resistance gene via allelic exchange. A plasmid carrying the *pasP* gene was introduced into the PASP-deficient mutant to construct a rescue strain. The PASP protein in the culture supernatants was determined by Western blot analysis. Corneal virulence was evaluated in rabbit and mouse keratitis models by slit lamp examination (SLE), bacterial enumeration, and/or histopathological analysis. Various host proteins and the rabbit tear film were analyzed for their susceptibility to PASP degradation.

RESULTS. The PASP-deficient mutant produced a significantly lower mean SLE score when compared with the parent or rescue strain ($P \leq 0.03$) at 29 hours postinfection (PI). All of the strains grew equally in the rabbit cornea ($P = 0.971$). Corneas infected with the PASP-deficient mutant showed moderate histopathology compared with those infected with the parent or rescue strain, which produced severe pathology inclusive of epithelial erosions, corneal edema, and neutrophil infiltration. In the mouse model, eyes inoculated with the PASP-deficient mutant had a significantly lower mean SLE score at 24 hours PI than the eyes inoculated with the parent or rescue strain ($P \leq 0.007$). PASP was found to degrade complement C3, fibrinogen, antimicrobial peptide LL-37, and constituents of the tear film.

CONCLUSIONS. PASP is a commonly secreted protease of *P. aeruginosa* that contributes significantly to the pathogenesis of keratitis.

Keywords: virulence, protease-deficient, *P. aeruginosa*, animal model, keratitis

Pseudomonas aeruginosa is an important opportunistic human pathogen that can cause infection at various body sites, including the cornea. In cases of bacterial keratitis that are contact lens associated, *P. aeruginosa* is the most common causative organism.^{1,2} *P. aeruginosa* keratitis is a rapidly progressive and destructive disease that can cause severe symptoms, such as ulcers, corneal perforation, and subsequent loss of vision.³

The pathogenesis of *P. aeruginosa* keratitis is complex and multifactorial, involving both bacterial factors and host components. As a result, eradication of the bacteria by antibiotics does not readily limit the pathological effects. Proteases produced by *P. aeruginosa* have long been studied as possible mediators of corneal damage and essential virulence factors during keratitis. Early studies with crude or purified *Pseudomonas* proteases demonstrated their toxicity to the rabbit cornea.^{4–6} Furthermore, a spectrum of proteins pertinent to host defense or tissue integrity was found to be susceptible to degradation by *Pseudomonas* proteases.^{7–9} The best evidence of a protease as an important virulence factor comes from studies of a protease-deficient mutant that, relative to the parent or rescue (complemented) strain, has reduced virulence but no decrease in its ability to grow in the rabbit cornea.

Among the well-studied *P. aeruginosa* proteases are the three metalloproteases: alkaline protease (AP), elastase A (LasA), and elastase B (LasB).^{10–12} Infection of animal corneas

with a mutant *P. aeruginosa* strain deficient in AP, LasA, or LasB has determined that none of these enzymes is essential for corneal virulence.¹³ The secretion of active *P. aeruginosa* protease has been achieved by cloning into *Pseudomonas putida*, the specific *P. aeruginosa* protease gene and its auxiliary genes needed for protease maturation and secretion. Because *P. putida* lacks a secreted protease and grows in the rabbit cornea without causing pathological effects, infection with *P. putida* secreting a *P. aeruginosa* protease can reveal the specific contribution of the cloned protease to corneal virulence. *P. putida* secreting LasB caused significantly greater corneal pathology than its parent strain harboring the plasmid vector alone.¹⁴ *P. putida* secreting AP was not significantly more virulent than its parent strain.¹⁴ Furthermore, *P. putida* secreting AP was significantly less virulent than the *P. putida* strain that secreted LasB.¹⁴ However, the importance of LasB to corneal infections is probably limited because multiple clinical strains of *P. aeruginosa* have been found to lack LasB expression.^{15,16} Also, a study by Kernacki et al.¹⁷ demonstrated that LasB was not produced in vivo during corneal infection with *P. aeruginosa*. Taken together, the results in these experimental models indicate that none of the three metalloproteases of *P. aeruginosa* likely plays a crucial role in the pathogenesis of keratitis.

P. aeruginosa also secretes a serine protease designated as protease IV (PIV). PIV has been demonstrated to be an

important virulence factor in the rabbit cornea.^{18,19} PIV is found in all clinical isolates analyzed, a finding that differs from AP and LasB.¹⁶ The loss of the *piv* gene has been shown to significantly reduce corneal virulence, and complementation (rescue) of this gene resulted in restored corneal virulence.¹⁶ Also, the secretion of PIV by *P. putida* resulted in substantial corneal virulence, significantly more than the *P. putida* parent strain.²⁰ PIV can degrade proteins of importance to host defense, including complement, fibrinogen, plasminogen, immunoglobulin, and surfactant proteins.^{9,21}

P. aeruginosa small protease (PASP) is a more recently identified protease.²² The *pasP* gene, like the gene for PIV, has been found in all *P. aeruginosa* isolates analyzed.²³ Injection of purified recombinant PASP into the rabbit cornea causes severe ocular pathology, including epithelial and stromal erosions, edema, and neutrophil infiltration into the corneal stroma.²³ PASP has also been shown to degrade host proteins important to the corneal structure, such as collagens.²³ Attempts to express PASP in *P. putida* did not achieve secretion of the active enzyme, indicating that yet to be identified auxiliary genes of *P. aeruginosa* are required for PASP secretion (Tang A, unpublished findings, 2008).

The present study investigated the virulence contribution of PASP by analyzing the virulence of the PASP-deficient mutant, its parent strain, and a rescue strain in both rabbit and mouse keratitis models. Additionally, the ability of PASP to cleave several key host proteins is described herein.

METHODS

Bacteria and Media

P. aeruginosa strain PA103-29 has been well characterized in the rabbit model of keratitis and as a source of PASP.^{16,23} PA103-29 is known to lack LasB and LasA production.²⁴ Bacteria were grown in tryptic soy broth (TSB; BD Biosciences, Sparks, MD) at 37°C with shaking. The inoculum for corneal infections was grown overnight in TSB, subcultured in fresh TSB, and grown to log phase (0.2–0.3 OD at 650 nm) before being diluted in TSB. The number of bacteria in the inoculum was verified by culturing dilutions of the bacteria on tryptic soy agar (TSA) in triplicate.

Construction of the PASP-Deficient Mutant and Its Rescue Strain

To construct the allelic exchange vector, as shown in Figure 1A, the flanking regions of the *pasP* gene and the *Tet* gene were PCR amplified with additions of restriction enzyme sites at both ends. These three PCR products were ligated and cloned into an intermediate plasmid, pGEM-T (Promega Corporation, Madison, WI). Then, the cassette containing the flanking regions and the *Tet* gene was excised and cloned into the suicidal vector pEX100T (kindly provided by Dr J. Hobden, Louisiana State University Health Sciences Center, New Orleans, LA), forming plasmid pEX100T-ΔPASP. The pEX100T vector expresses the *sacB* gene (levansucrase) that renders the host bacterium susceptible to sucrose. Introduction of pEX100T-ΔPASP into *P. aeruginosa* was accomplished by conjugal mating.²⁵ Briefly, *Escherichia coli* carrying pEX100T-ΔPASP was mated with the *E. coli* strain harboring the conjugal plasmid (pRK2013) to generate a strain with both plasmids. Subsequently, this *E. coli* strain was mated with PA103-29 (recipient strain) and the transconjugants were selected on *Pseudomonas* isolation agar (BD Biosciences) supplemented with 10% sucrose and 150 μg/mL tetracycline.

To construct the rescue strain, the *pasP* gene of PA 103-29 was cloned into the *Pseudomonas/E. coli* shuttle vector pUCP20, conferring carbenicillin resistance (Fig. 1B). The resulting pUCP20-*pasP* plasmid was introduced into the PASP-deficient mutant by chemical transformation as previously described.²⁰

PCR Analysis and DNA Sequencing

DNA from a *P. aeruginosa* colony was used as the template for PCR and the reactions for the *pasP* and *Tet* genes were conducted under routine PCR conditions (94°C for 1 minute; then 30 cycles of 94°C for 20 seconds, 55°C for 20 seconds, 68°C for 1 minute). To further confirm the correct replacement of the *pasP* gene of the PASP-deficient mutant, the affected chromosomal region was PCR-amplified and cloned into pGEM-T for sequencing. This chromosomal amplicon (~4 kb) covering additional 150 bp outside the exchange cassette was sequenced in both directions (SeqWright, Inc., Houston, TX).

Western Blot Analysis

Pseudomonas cultures were grown for 30 hours in M9 minimum medium as previously described.⁹ For plasmid maintenance, the M9 medium was supplemented with 100 μg/mL carbenicillin. Bacteria were removed by centrifugation and the supernatant was filtered through a 0.22-μm filter, and concentrated 50-fold using an ultrafiltration cell (Amicon; Millipore, Billerica, MA) with a 10-kDa molecular weight cutoff filter. Western blot analysis on the concentrated culture supernatants was performed as previously described.²³

Rabbit Intrastromal Injection Model of Keratitis

Specific pathogen-free New Zealand white rabbits were obtained from Charles River Laboratories International, Inc. (Wilmington, MA). Rabbits were maintained according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rabbits were anesthetized by subcutaneous injection of a mixture of xylazine (100 mg/mL; Butler Co., Columbus, OH) and ketamine (100 mg/mL; Fort Dodge Animal Health, Fort Dodge, IA). Proparacaine (Bausch and Lomb, Tampa, FL) was topically applied to the eyes before injections. *Pseudomonas* cultures were grown to log phase and 1000 colony-forming units (CFU) of bacteria in 10 μL TSB were intrastromally injected into rabbit corneas.

Mouse Topical Inoculation Model of Keratitis

Male C57/B6 mice, 4 to 5 weeks old, were obtained from Harlan Laboratories, Inc. (Indianapolis, IN). To prepare the ketamine solution for mouse anesthesia, a 1.5-mL volume of xylazine was mixed with 10 mL ketamine and diluted 1:4 with saline. Mice were subcutaneously injected with this solution at a dose of 0.1 mL/20 g body weight. The mouse keratitis protocol was developed based on methods described by Kwon and Hazlett,²⁶ Girgis et al.,²⁷ and McCormick et al.²⁸ *Pseudomonas* cultures were grown to mid-log phase (OD₆₅₀ = 0.35, approximately 2×10^8 /mL). Mouse corneas were scarified by making four to six parallel abrasions in the corneal epithelium using a 30.5-gauge needle. An inoculum with 10⁶ CFU bacteria in 5 μL TSB was topically applied to each scarified eye.

Slit Lamp Examination

Rabbit eyes ($n \geq 8$) underwent slit lamp examination (SLE) at 29 hours postinfection (PI) by two masked observers. Each of

A

Plasmid

Chromosome

P. aeruginosa

B

FIGURE 1. Construction of the allelic exchange construct, pEX100T- Δ PASP, and rescue plasmid pUCP20-*pasP*. (A) A cassette containing the flanking regions of the *pasP* gene and the tetracycline resistance gene *Tet* was assembled and cloned into the suicidal vector pEX100T. The replacement of the *pasP* gene in the chromosome with the *Tet* gene resulted from homologous recombination. *sacB*, levansucrase. (B) To construct the rescue plasmid, the *pasP* gene was PCR-amplified with addition of the restriction sites *EcoRI* and *BamHI* at the 5' and 3' end, respectively. Then, the *pasP* gene was cloned into the *Pseudomonas/E. coli* shuttle vector pUCP20 under the *lacZ* promoter. *lacZ*, β -galactosidase; *bla*, β -lactamase.

the seven parameters (injection, chemosis, iritis, hypopyon, corneal infiltrate, fibrin in the anterior chamber, corneal edema) was graded on a scale ranging from 0 (normal) to 4 (severe), as described by O'Callaghan et al.²⁹ The sum of these grades for an eye, after averaging, determined the SLE score, which could range from 0 to a theoretical maximum of 28.

Mouse eyes ($n \geq 8$) were scored at 24 hours PI on the following scale: 0, clear and normal; 1, light corneal haze; 2, readily detectable corneal opacity; 3, infiltrate partially covering the pupil; 4, infiltrate covering the entire pupil and an ulcer fully formed; and 5, corneal perforation or panophthalmitis.²⁸ Mean SLE scores ($\pm$ SEM) were calculated.

Quantification of Viable Bacteria

Rabbit corneas ($n \geq 4$) were harvested at 29 hours PI and homogenized in 3.0 mL sterile PBS. A 0.1-mL aliquot of the homogenate was serially diluted (1:10) in PBS and plated on TSA in triplicate.

Whole mouse eyes ($n \geq 4$) were harvested at 24 hours PI and individually homogenized in 1.0 mL PBS. Mouse eye homogenates were diluted in PBS and cultured on *Pseudomonas* isolation agar. CFU was determined and expressed as base 10 logarithms.

Histopathology

Rabbit corneas were harvested at 29 hours PI ($n \geq 3$). After fixation, specimens were processed by Excalibur Pathology,

Inc. (Oklahoma City, OK). The tissue sections were stained with hematoxylin and eosin. Each slide was examined microscopically and photographed under low magnification ($\times 40$).

Degradation of Host Proteins and Tears

Recombinant PASP (rPASP) and native PIV were purified as previously described by Tang et al.²³ and Engel et al.,⁹ respectively. Tears were collected using capillary tubes placed into the cul-de-sac of normal rabbit eyes. Human host proteins, including fibrinogen (Sigma-Aldrich, St. Louis, MO), complement C3 (Calbiochem-Novabiochem Corp., La Jolla, CA), and antimicrobial peptide LL-37 (Phoenix Pharmaceuticals, Inc., Burlingame, CA), as well as rabbit tears, were incubated with dH₂O, active rPASP, an equal volume of heat-inactivated rPASP, or PIV at 37°C for various periods of time. Aliquots of the reaction mixtures were electrophoresed on 12% SDS-acrylamide gels and stained with Coomassie blue. The degradation of proteins was indicated by changes in electrophoretic mobility of protein components.

Statistical Analysis

Mean and SEM were calculated using Microsoft Excel (Microsoft Corporation, Redmond, WA). For comparison of SLE scores, nonparametric one-way analysis of variance (Kruskal-Wallis test) was performed.²⁹ Student's *t*-test was used for CFU determination data. *P* less than or equal to 0.05 was considered significant.

RESULTS

Confirmation of the PASP-Deficient Mutant and Its Rescue Strain

The entire *pasP* gene of PA103-29 was replaced with the tetracycline resistance gene *Tet* via allelic exchange (Fig. 1A). This exchange event was confirmed by sequencing the affected chromosomal region from base 469,356 to base 472,785 (*pasP*: 470,662–471,237). There was no unintended change found in the flanking regions of the *pasP* gene. To construct the rescue strain, the *pasP* gene of PA103-29 was cloned into the expression vector pUCP20, forming a plasmid designated as pUCP20-*pasP* (Fig. 1B). The construct pUCP20-*pasP* was confirmed by DNA sequencing. Plasmid pUCP20-*pasP* was introduced by transformation into the mutant (PA103-29Δ*PASP*), forming a rescue strain.

PCR analysis was performed on the parent, PASP-deficient, and rescue strains to verify the presence or absence of the *pasP* and *Tet* genes. As expected, the *pasP* gene was detected in the parent and rescue strains, whereas the *Tet* gene was detected in the PASP-deficient and rescue strains (Fig. 2A). In vitro, the PASP-deficient mutant grew as well as the parent or rescue strain; and the rescue strain, under carbenicillin selection (100 μg/mL), grew as well as the other two strains without antibiotic selection (data not shown).

To analyze the production and secretion of the PASP protein, concentrated culture supernatants of the three strains were subjected to Western blot analysis (Fig. 2B). The PASP band of correct size (19 kDa) appeared only in the supernatants of the parent and rescue strains, but not in that of the PASP-deficient mutant.

Virulence Analysis of the PASP-Deficient Mutant in Animal Keratitis Models

To examine the virulence contribution of PASP, experimental bacterial keratitis was initiated in both rabbits and mice by inoculating their corneas with the parent, PASP-deficient

mutant, or rescue strain. SLE, bacteria enumeration, and/or histopathological analysis were performed in these animal studies.

Rabbit Intrastromal Injection Model of *P. aeruginosa* Keratitis. Intrastromal injection into the rabbit cornea of 1000 CFU of log-phase parent strain, PASP-deficient mutant, or rescue strain caused apparent pathology in all rabbit eyes at 29 hours PI. However, the disease in the eyes injected with the PASP-deficient mutant appeared milder than that of the eyes injected with the parent or rescue strain (Fig. 3A). The eyes injected with the parent or rescue strain, as compared with eyes injected with the PASP-deficient mutant, demonstrated more pronounced conjunctival swelling, corneal infiltration, and epithelial erosions. Also noted is that, at 16 hours PI, all of the infected rabbit eyes looked similar.

At 29 hours PI, the eyes injected with the parent, PASP-deficient, or rescue strain produced a mean SLE score of 14.58 ± 1.09 , 9.25 ± 1.49 , or 17.75 ± 1.39 , respectively (Fig. 3B). The SLE score of the eyes injected with the mutant was significantly lower as compared with the score of the eyes injected with the parent ($P = 0.03$) or the rescue strain ($P = 0.011$). The SLE score of the eyes injected with the rescue strain was not significantly higher than that of the eyes injected with the parent strain ($P = 0.109$). The parent, PASP-deficient, and rescue strains grew equally, reaching 7.23 ± 0.09 , 7.11 ± 0.27 , or 7.40 ± 0.08 log CFU at 29 hours PI, respectively ($P = 0.971$).

The infected corneas underwent histopathological analysis at 29 hours PI (Fig. 4). In the corneas injected with the parent or rescue strain, the erosion of the corneal epithelium, the infiltration of neutrophils into the corneal stroma, and corneal

FIGURE 2. Analysis of the PASP gene and protein. (A) Colony PCR analysis of PA103-29, the PASP-deficient mutant, and its rescue strain for the presence of the *Tet* and *pasP* genes. A single colony of PA103-29 (parent), PA103-29Δ*PASP* (mutant), or PA103-29Δ*PASP*/pUCP20-*pasP* (rescue) was suspended in 30 μL dH₂O and boiled for 5 minutes. Aliquots of the supernatants of the colony suspensions (5 μL added to a 25-μL PCR reaction) were used as template DNA. (B) Identification of the PASP protein in the concentrated *Pseudomonas* culture supernatants. Concentrated culture supernatants of PA103-29 (20 μL, 3 mg/mL total protein), PA103-29Δ*PASP* (20 μL, 3 mg/mL), and PA103-29Δ*PASP*/pUCP20-*pasP* (20 μL, 3 mg/mL) were analyzed by Western blot analysis. Lane 1: parent strain; lane 2: PASP-deficient mutant; and lane 3: rescue strain.

FIGURE 3. Evaluation of ocular virulence of the PASP-deficient mutant and its parent and rescue strains in a rabbit intrastromal injection model of keratitis. (A) Photographs of rabbit eyes at 29 hours after intrastromal injection of the three strains. (B) SLE scores of infected rabbit eyes at 29 hours PI. The mean SLE score of the mutant group is significantly lower than that of the parent group ($*P = 0.03$) or the rescue group ($\dagger P = 0.011$). There is no difference in SLE scores between the parent group and the rescue group ($\ddagger P = 0.109$).

FIGURE 4. Histopathological analysis of rabbit corneas infected with the parental strain PA103-29, the PASP-deficient mutant, or the rescue strain. Corneas ($n \geq 3$) were harvested at 29 hours PI. Corneal sections were stained with hematoxylin and eosin, microscopically examined, and photographed under low power ($\times 40$). The parent and rescue strains caused extensive epithelial erosion (detachment), severe neutrophil infiltration, and corneal edema. In contrast, the PASP-deficient mutant-injected corneas showed no or trace epithelial erosion, mild neutrophil infiltration, and no edema.

edema were much more severe than that caused by the PASP-deficient mutant.

Mouse Scratch Model of *P. aeruginosa* Keratitis. The eyes of C57/B6 mice were scarified and topically inoculated with 1×10^6 CFU of the strains. All three strains caused pathology in the mouse eyes by 24 hours after inoculation. However, the eyes inoculated with the PASP-deficient mutant demonstrated significantly less pathology than those eyes inoculated with the parent or rescue strain (Fig. 5A). The eyes inoculated with the parent or rescue strain had extensive corneal infiltration covering more than three-quarters of the cornea, whereas the infiltrate in the eyes inoculated with the PASP-deficient mutant was light, covering less than half of the cornea. The mouse eyes inoculated with the PASP-deficient mutant had a significantly lower SLE score than the eyes

inoculated with the parent ($P = 0.009$) or rescue strain ($P = 0.007$) at 24 hours PI (Fig. 5B). The bacterial load for all three strains in the mouse eyes at 24 hours PI was equivalent, approximately 5.22 ± 0.08 , 5.32 ± 0.14 , or 5.54 ± 0.14 log CFU for the eyes inoculated with the parent, PASP-deficient, or rescue strain, respectively ($P = 0.990$). At 48 hours PI, mouse eyes of all three groups showed similar pathology and contained more than 6 logs CFU. By 72 hours PI, all eyes had more than 7 logs CFU and the pathology was severe in all three groups.

PASP Effects on Host Proteins and the Rabbit Tear Film

Pseudomonas proteases are known to degrade some important components of the host defense system, such as complement and immunoglobulin.^{7,9} The degradation of several host defense molecules by PASP was determined by changes in electrophoretic mobility of protein components during SDS-PAGE. PASP was found to degrade fibrinogen α - and β -chains, but not γ -chain, after a 4-hour incubation (Fig. 6A). PASP degraded fibrinogen fully after a longer incubation (24 hours; data not shown). Human complement C3 α -chain was also susceptible to PASP degradation (Fig. 6B). Both PASP and PIV degraded the antimicrobial peptide LL-37 (Fig. 6C). When incubated with rabbit tears, PASP and PIV digested two ranges of tear proteins, one range above 80 kDa and the other between 25 and 45 kDa (Fig. 6D). Among the host proteins tested, none of the immunoglobulin types (IgA, IgG, and IgM) was susceptible to PASP degradation, and neither was fibrin, complement C1q, plasmin, or plasminogen (data not shown).

DISCUSSION

The role of PASP in corneal virulence has been investigated in the present study by analyzing the virulence of the PASP-deficient mutant in two animal models of *P. aeruginosa* keratitis. The PASP-deficient mutant demonstrated significantly reduced virulence in both the rabbit intrastromal injection model and the mouse scratch model of keratitis. The PASP-deficient mutant in both models grew as well as the parent or rescue strain, but produced a significantly lower SLE score. Both histological analysis and gross examination of the eyes infected with the PASP-deficient mutant showed less inflammation and tissue damage than that of eyes infected with either the parent or rescue strain. PASP was found to degrade complement C3 and antimicrobial peptide LL-37, two members of the innate host defense system. Like PIV, PASP was also able to degrade multiple proteins of the rabbit tear film.

FIGURE 5. Virulence comparison of the PASP-deficient mutant, its parent strain, and the rescue strain in a mouse topical inoculation model of keratitis. (A) Photographs of infected mouse eyes at 24 hours PI. (B) SLE scores of infected mouse eyes. The mean SLE score of the mutant group is significantly lower than that of the parent group ($*P = 0.009$) or the rescue group ($†P = 0.007$). There is no difference in SLE scores between the parent group and the rescue group ($‡P = 0.899$).

FIGURE 6. Degradation of host proteins and the tear film by PASP. Heat-inactivated (lane 1) or active rPASP (1 μ g; lane 2) was incubated with fibrinogen (A) or complement C3 (B) at 37°C for 4 hours. Antimicrobial peptide LL-37 (C) and rabbit tears (D) were incubated with dH₂O (lane 1), active rPASP (1 μ g; lane 2), or PIV (1 μ g; lane 3) for 14 hours and 5 hours, respectively. The arrows indicate where the changes in electrophoretic mobility of the host protein components occurred.

The construction of the PASP-deficient mutant was achieved through allelic exchange with the entire open reading frame of the *pasP* gene being deleted and replaced with the *Tet* gene. Sequencing data confirmed the *Tet* replacement of the *pasP* gene occurred without any unintended DNA changes in the regions neighboring the *pasP* gene. Hence, the PASP-deficient mutant is a true isogenic mutant of PA103-29. As demonstrated by Western blot analysis, the PASP protein was absent in the culture supernatant of the mutant strain, but was present in culture supernatants of both the parent and rescue strains. Thus, the expected phenotypes of the strains were demonstrated.

The present research has demonstrated that the deletion of the *pasP* gene did not alter the bacterial growth in vitro, or in vivo in the rabbit or mouse cornea. In the rabbit model, the rescue strain produced a higher SLE score than the parent strain, although it was not a statistically significant difference. There is a possibility that the increased production of PASP, which could be due to the multiple copies of the rescue plasmid in a cell, could have enhanced the virulence of the rescue strain. Previously, Caballero et al.¹⁶ showed that a PIV-deficient mutation resulted in significantly reduced virulence in the rabbit model of keratitis, whereas a rescue strain had virulence equivalent to the parent strain PA103-29. When considered together, the findings of the Caballero et al. study¹⁶ and the present study suggest that PIV and PASP are both important corneal virulence factors, but they cannot substitute for each other to achieve full corneal virulence when one protease is missing. Studies of PASP coupled with those of PIV, strongly support the hypothesis that *Pseudomonas* proteases play a major role in keratitis. These two enzymes, unlike other *Pseudomonas* proteases, are the focus of this consideration because they are found in all strains tested.^{16,23,30}

There are now some ideas that could explain what the proteases accomplish during infection. PASP, unlike PIV, has a potent action on collagens, thus mediating the loss of epithelial cells and the formation of an ulcer extending down into the corneal stroma.²³ The elimination of epithelial cells may remove a potent source of proinflammatory cytokines and other defensive molecules (e.g., LL-37). PIV is not efficient in cleaving collagens and does not cause erosions when expressed in nonpathogenic *P. putida* during experimental keratitis.^{20,23} However, PIV, like PASP, can cleave both C3 and LL-37, two very active members of the host defense system. PIV has also been demonstrated to digest surfactant proteins, and these molecules have been shown to protect the cornea from *Pseudomonas*

infection.^{21,28,31} Complement mediates a protective inflammatory response that delivers both cellular and humoral components of the host defense system to the site of infection, so a reduction in the available pool of complement is considered a means to reduce the host defense against infection. Both C3 and LL-37 facilitate phagocytosis of bacterial pathogens, so the proteolytic destruction of these proteins could favor survival of the bacteria at the site of infection.^{32,33} LL-37 is also a proven nonspecific defensive molecule for the cornea that has bactericidal activity. LL-37, which corresponds to the C-terminal 37 amino acids (4.5 kDa) of the human antimicrobial protein cathelicidin (hCAP18), can form pores in bacterial cell membranes resulting in cell death.³⁴ LL-37 is produced by neutrophils and macrophages, and, very importantly, by epithelial cells of the cornea.³⁵ Huang et al.³⁶ reported that mice deficient in cathelicidin exhibited increased susceptibility to *P. aeruginosa* keratitis, indicating the importance of LL-37 in defending the cornea from microbial infections.

Degradation of fibrinogen, but not fibrin, by PASP may delay corneal wound healing by reducing the source of fibrin whose deposition in a wound is one of the initiating events in wound healing.³⁷ *P. aeruginosa* primarily infects compromised tissue and corneal epithelial defects compromise the cornea to *P. aeruginosa* keratitis. By limiting corneal healing, a corneal defect could persist instead of being rapidly healed. Another aspect of wound healing that is inhibited by *Pseudomonas* proteases is the cleavage of plasminogen by PIV.⁹ PIV, but not PASP, has been demonstrated to degrade plasminogen, thus removing the source of plasmin. Plasmin, a serine protease, is used to dissolve the meshwork of fibrin formed during wound healing.³⁷ By digesting plasminogen, PIV possibly deprives the compromised tissue site of plasmin, thus delaying the process of normal wound healing.

Although there are some similarities between the activities of PASP and PIV, there are some important differences that could explain why one enzyme cannot substitute for the other. Both can cleave C3 and LL-37 and both digested tear constituents in a similar manner. Among the differences between the two proteases is the ability of PIV, but not PASP, to cleave immunoglobulin and plasminogen, two molecules that contribute to the host defense system. However, PASP, unlike PIV, can cleave collagens favoring the loss of the epithelium and the formation of a corneal erosion. Thus, PIV and PASP share some activities, but there are important reactions contributing to virulence that can be performed by only one of these enzymes.

Previous studies have shown that TLCK, a serine protease inhibitor, can inhibit both PIV and PASP activity.^{9,23} Unfortunately, TLCK is highly toxic to the eye and cannot be used as therapy in vivo. Because both proteases are important corneal virulence factors, there would be benefit in using an effective serine protease inhibitor as part of the therapy for *Pseudomonas* keratitis. In the past, researchers have tested a synthetic matrix metalloprotease inhibitor to treat *P. aeruginosa* keratitis.³⁸ Studies also have shown that proteases of *P. aeruginosa* can activate host matrix metalloproteases and lead to host-derived damaging effects.^{39–41} As a result, this matrix metalloprotease inhibitor effectively inhibited the metalloproteases produced by *P. aeruginosa*, and topical treatment with the inhibitor prevented the corneal destruction caused by injection of sterile culture filtrate. However, when live bacteria were injected, the inhibitor could not prevent the corneal damage.³⁸ These findings suggest that a protease inhibitor, or a cocktail of inhibitors, with a focus on serine protease inhibitors, could work better in preventing corneal pathology.

The main finding of the present study is that the isogenic PASP mutant causes significantly reduced pathology in the rabbit or mouse cornea. PASP is also found to degrade proteins important to the corneal structure and in host defense. In conclusion, PASP is a commonly produced protease of *P. aeruginosa* that is essential for full corneal virulence.

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